

**MAMMALIAN ALPHA-KINASE PROTEINS, NUCLEIC ACIDS  
AND DIAGNOSTIC AND THERAPEUTIC USES THEREOF**

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RELATED APPLICATIONS

The present application is a continuation-in-part of copending application Serial No. 09/632,131 filed August 3, 2000, of which the instant application claims the benefit of  
10 the filing date pursuant to 35 U.S.C. § 120, and which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

15 This invention relates generally to the identification of a new superfamily of eukaryotic protein alpha kinases, and particularly to members of a subfamily selected from the group of melanoma alpha kinase, kidney alpha kinase, heart alpha kinase, skeletal muscle alpha kinase and lymphocyte alpha kinase. The invention further relates to the use of the alpha kinases in assays to screen for specific modulators  
20 thereof. Isolated nucleic acids encoding the alpha kinases - melanoma alpha kinase, kidney alpha kinase, heart alpha kinase, skeletal muscle alpha kinase and lymphocyte alpha kinase - are provided herein.

BACKGROUND OF THE INVENTION

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Protein phosphorylation plays a critical role in many cellular processes (Krebs (1994) *Trends Biochem. Sci.* 19:439; Hanks and Hunter, (1996) *FASEB J.* 9:576-596; Hardie and Hanks, (1995) *The Protein Kinase Facts Book* (Academic, London)). There are two well-characterized superfamilies of protein kinases, with most of the protein  
30 kinases belonging to the serine/threonine/tyrosine kinase superfamily (Hanks and Hunter, (1996); Hardie and Hanks, (1995)). The characterization of several hundred

members of this superfamily revealed that they all share a similar structural organization of their catalytic domains which consist of twelve conserved subdomains (Hanks and Hunter, (1996); Hardie and Hanks, (1995)). The other superfamily is referred to as the histidine kinase superfamily and is involved in the prokaryotic two-

5 component signal transduction system, acting as sensor components (Stock et al., (1989) *Microbiol. Rev.* 53:450-490; Parkinson and Kofoed, (1992) *Annu. Rev. Genet.* 26:71-112; Swanson, et al., (1994) *Trends Biochem. Sci.* 19:485-490). Recently, eukaryotic members of this superfamily have also been described (Chang et al., (1993) *Science* 263:539-544; Ota and Varshavsky, (1993) *Science* 262:566-569;

10 Maeda et al., (1994) *Nature* 369:242-245). Mitochondrial protein kinases have also recently been described that show structural homology to the histidine kinases, but phosphorylate their substrates on serine (Popov et al., (1992) *J. Biol. Chem.* 267:13127-13130; Popov et al., (1993) *J. Biol. Chem.* 268:26602-22606). Finally, several new protein kinases have been reported that show a lack of homology with

15 either of the kinase superfamilies (Maru and Witte, (1991) *Cell* 67:459-468; Beeler et al., (1994) *Mol. Cell. Biol.* 14:982-988; Dikstein et al., (1996) *Cell* 84:781-790; Futey et al., (1995) *J. Biol. Chem.* 270:523-529; Eichenger et al., (1996) *EMBO J.* 15:5547-5556). However, these protein kinases are viewed as an exception to the general rule as they have yet to be fully characterized.

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The cloning and sequencing of the extensively characterized eukaryotic elongation factor-2 kinase (eEF-2 kinase) from a variety of eukaryotic organisms has revealed the existence of a novel class of protein kinases (Ryazanov et al., (1997) *Proc. Natl. Acad. Sci., USA* 94:4884-4889). eEF-2 kinase, previously known as Ca<sup>2+</sup>/calmodulin-

25 dependent protein kinase III, is highly specific for phosphorylation of elongation factor-2 (eEF-2), an abundant cytoplasmic protein that catalyzes the movement of the ribosome along mRNA during translation in eukaryotic cells (reviewed in Ryazanov and Spirin, (1993) In *Translational Regulation of Gene Expression* (Plenum, New York) Vol. 2, pp. 433-455; Nairn and Palfrey, (1996) In *Translational Control*

30 (CSHL Press, New York) pp. 295-318). All mammalian tissues, and various

invertebrate organisms, exhibit eEF-2 kinase activity (Abdelmajid et al., (1993) *Int. J. Dev. Biol.* 37:279-290). eEF-2 kinase catalyzes the phosphorylation of eEF-2 at two highly conserved threonine residues located within a GTP-binding domain (Ryazanov and Spirin, (1993) In *Translational Regulation of Gene Expression* (Plenum, New York) Vol. 2, pp. 433-455; Nairn and Palfrey, (1996) In *Translational Control* (CSHL Press, New York) pp. 295-318). When eEF-2 is phosphorylated, it becomes inactive with respect to protein synthesis (Ryazanov et al., (1988) *Nature* 334:170-173). Since eEF-2 phosphorylation is dependent on  $\text{Ca}^{2+}$  and calmodulin, eEF-2 kinase plays a pivotal role in modulating the protein synthesis rate in response to changes in intracellular calcium concentration. Phosphorylation of eEF-2 has also been linked to the regulation of cell cycle progression. For example, transient phosphorylation of eEF-2 occurs during the mitogenic stimulation of quiescent cells (Palfrey et al., (1987) *J. Biol. Chem.* 262:9785-9792) and during mitosis (Celis et al., (1990) *Proc. Natl. Acad. Sci., USA* 87:4231-4235). In addition, changes in the level of eEF-2 kinase activity is associated with a host of cellular processes such as cellular differentiation (End et al., (1982) *J. Biol. Chem.* 257:9223-9225; Koizumi et al., (1989) *FEBS Lett.* 253:55-58; Brady et al., (1990) *J. Neurochem.* 54:1034-1039), oogenesis (Severinov et al., (1990) *New Biol.* 2: 887-893), and malignant transformation (Bagaglio et al., (1993) *Cancer Res.* 53:2260-2264).

The sequence of eEF-2 kinase appears to have no homology to either the  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinases or to any members of the known protein kinase superfamilies (Ryazanov et al., (1997) *Proc. Natl. Acad. Sci., USA* 94:4884-4889). However, the recently described myosin heavy chain kinase A (MHCK A) from *Dictyostelium* (Futey et al., (1995) *J. Biol. Chem.* 270:523-529) shows a great deal of homology with eEF-2 kinase. These two kinases define a novel class of protein kinases that may represent a new superfamily.

Evidence for MHCK and eEF-2 kinase forming the core of a new superfamily is as follows. MHCK A from *Dictyostelium*, has a demonstrated role in the regulation of

myosin assembly (Futey et al., (1995) *J. Biol. Chem.* 270:523-529; Côté et al., (1997) *J. Biol. Chem.* 272:6846-6849). eEF-2 kinase is a ubiquitous  $\text{Ca}^{2+}$ /calmodulin-dependant protein kinase involved in the regulation of protein synthesis by  $\text{Ca}^{2+}$  (Redpath et al., (1996) *J. Biol. Chem.* 271:17547-17554; Ryazanov et al., (1997) *Proc. Natl. Acad. Sci., USA* 94:4884-4889). Both MHCK A and eEF-2 kinase display no  
 5 homology to any of the known protein kinases, but are strikingly similar to each other; amino acid sequences of their catalytic domains are 40% identical. Another protein kinase homologous to MHCK A and eEF-2 kinase has recently been identified in *Dictyostelium* (Clancy et al., (1997) *J. Biol. Chem.* 272:11812-11815), and an  
 10 expressed sequence tag (EST) sequence, with a high degree of similarity to the catalytic domain common to both MHCK A and eEF-2 kinase, has been deposited in GenBank (clone FC-AN09/accession #C22986). An amino acid sequence alignment of the catalytic domains of these new protein kinases is shown in Figure 1A. These  
 15 kinases have a catalytic domain of approximately 200 amino acids which can be subdivided into seven conserved subdomains. Subdomains V, VI, and VII have a predicted  $\beta$ -sheet structure and are presumably involved in ATP-binding, while subdomains I through IV may be involved in substrate binding and catalysis. These new protein kinases have no homology to the members of the eukaryotic  
 20 serine/threonine/tyrosine protein kinase superfamily with the exception of the GXGXXG motif in subdomain VI which is present in many ATP-binding proteins. Thus, MHCK A, eEF-2 kinase, and related protein kinases may represent a new superfamily. Evolutionary analysis of these new kinases (Fig. 1B) reveals that they can be subdivided into 2 families: the eEF-2 kinase family which includes eEF-2  
 25 kinases from different organisms, and the MHCK family which includes MHCK A, MHCK B and FC-AN09. These two families appear to have split more than a billion years ago.

An interesting question is why does nature employ these unusual kinases to phosphorylate eEF-2 and myosin heavy chains? Perhaps the answer is related to the  
 30 secondary structure of the phosphorylation sites. As was originally reported by Small

et al. (Small et al., (1977), *Biochim. Biophys. Res. Comm.* 79:341-346), phosphorylation sites are usually located at predicted  $\beta$ -turns. Subsequent studies, including X-ray crystallographic data, demonstrated that phosphoacceptor sites in substrates of conventional protein kinases are often located in turns or loops and usually have flexible extended conformation (Knighton et al., (1991) *Science* 253:414-420; Pinna and Ruzzene (1996) *Biochim. Biophys. Acta* 1314:191-225). In contrast to this, the existing evidence suggests that the peptides around phosphorylation sites for eEF-2 kinases and MHCK A have an  $\alpha$ -helical conformation. The two major phosphorylation sites for MHCK A are located in a region which has a coiled-coil  $\alpha$ -helical structure (Vaillancourt et al., (1988) *J. Biol. Chem.* 253:10082-10087). The major phosphorylation site in eEF-2, threonine 56, is located within a sequence which is homologous among all translational elongation factors. In the crystal structure of the prokaryotic elongation factor EF-Tu, this sequence has an  $\alpha$ -helical conformation (Polekhina et al., (1996) *Structure* 4:1141-1151; Abel et al., (1996) *Structure* 4:1153-1159). These facts suggest that eEF-2 kinase and MHCK A differ from conventional protein kinases in that they phosphorylate amino acids located within  $\alpha$ -helices. Thus, in addition to the two well-characterized superfamily of eukaryotic protein kinases, which phosphorylate amino acids located in loops and turns, there appears to be a third superfamily of  $\alpha$ -helix-directed kinases.

The existence of several protein kinases which have very little or no homology to either the serine/threonine/tyrosine kinase superfamily or the histidine kinase superfamily, provides a new superfamily, the  $\alpha$ -kinases. The isolation and analysis of additional members of this family of kinases will further our understanding of  $\alpha$ -kinases and provide insight into the physiological roles of these kinases and their applications and uses.

The citation of references herein shall not be construed as an admission that such is prior art to the present invention.

### SUMMARY OF THE INVENTION

In accordance with the present invention, a new superfamily of protein kinases, novel  
 5 members thereof, and corresponding methods for assaying their phosphorylation  
 activity are disclosed. The protein kinases of this new alpha-kinase superfamily have  
 the following characteristics: 1) No significant sequence homology to protein kinases  
 of either the serine/threonine/tyrosine kinase or histidine kinase super families; 2)  
 moderate to high homology ( $\geq 40\%$ ) to eEF-2 kinases from any organism; and, 3) the  
 10 ability to phosphorylate an amino acid within an  $\alpha$ -helical domain. In addition, a new  
 subfamily of alpha-kinases is herein provided. In particular, a subfamily of alpha-  
 kinases is provided in which an ion channel, particularly belonging to the TRP family  
 of ion channels is covalently linked to a protein kinase. The placement of a kinase  
 and channel on a single molecule is particularly interesting and suggests a self-  
 15 regulated molecule, whereby the phosphorylation/autophosphorylation of these unique  
 alpha kinases controls or contributes to the open or closed state of the channel.

The present invention provides an isolated nucleic acid encoding melanoma alpha  
 kinase, or a fragment thereof having at least 15 nucleotides. In particular, the  
 20 invention provides an isolated nucleic acid encoding human melanoma alpha kinase,  
 wherein the nucleic acid is selected from the group consisting of :

- a. the DNA sequence of SEQ ID NO: 26;
- b. DNA sequences that hybridize to the sequence of subpart (a) under  
 moderate stringency hybridization conditions;
- 25 c. DNA sequences capable of encoding the amino acid sequence encoded  
 by the DNA sequences of (a) or (b);
- d. degenerate variants thereof;
- e. alleles thereof; and
- f. hybridizable fragments thereof.

In particular, the invention provides an isolated nucleic acid encoding mouse melanoma alpha kinase, wherein the nucleic acid is selected from the group consisting of :

- a. the DNA sequence of SEQ ID NO: 28;
- 5 b. DNA sequences that hybridize to the sequence of subpart (a) under moderate stringency hybridization conditions;
- c. DNA sequences capable of encoding the amino acid sequence encoded by the DNA sequences of (a) or (b);
- d. degenerate variants thereof;
- 10 e. alleles thereof; and
- f. hybridizable fragments thereof.

In particular, the invention provides an isolated nucleic acid encoding mammalian melanoma alpha kinase, wherein the nucleic acid is selected from the group consisting

- 15 of :
- a. the DNA sequence of SEQ ID NO: 28;
- b. the DNA sequence of SEQ ID NO: 26;
- c. DNA sequences that hybridize to the sequence of subparts (a) or (b) under standard hybridization conditions; and
- 20 d. DNA sequences capable of encoding the amino acid sequence encoded by the DNA sequences of subparts (a), (b) or (c).

The present invention further provides an isolated nucleic acid encoding heart alpha kinase, or a fragment thereof having at least 15 nucleotides. In particular, the present invention provides an isolated nucleic acid encoding human heart alpha kinase, wherein the nucleic acid is selected from the group consisting of :

- a. nucleic acid comprising the DNA sequence of SEQ ID NO: 34;
- 30 b. DNA sequences that hybridize to the sequence of subpart (a) under moderate stringency hybridization conditions;

- c. DNA sequences capable of encoding the amino acid sequence encoded by the DNA sequences of (a) or (b);
- d. degenerate variants thereof;
- e. alleles thereof; and
- 5 f. hybridizable fragments thereof.

In particular, the present invention provides an isolated nucleic acid encoding mouse heart alpha kinase, wherein the nucleic acid is selected from the group consisting of :

- a. nucleic acid comprising the DNA sequence of SEQ ID NO: 36;
- 10 b. DNA sequences that hybridize to the sequence of subpart (a) under moderate stringency hybridization conditions;
- c. DNA sequences capable of encoding the amino acid sequence encoded by the DNA sequences of (a) or (b);
- d. degenerate variants thereof;
- 15 e. alleles thereof; and
- f. hybridizable fragments thereof.

In particular, the invention provides an isolated nucleic acid encoding mammalian heart alpha kinase, wherein the nucleic acid is selected from the group consisting of :

- 20 a. the DNA sequence of SEQ ID NO: 34;
- b. the DNA sequence of SEQ ID NO: 36;
- c. DNA sequences that hybridize to the sequence of subparts (a) or (b) under standard hybridization conditions; and
- d. DNA sequences capable of encoding the amino acid sequence encoded
- 25 by the DNA sequences of subparts (a), (b) or (c).

The present invention still further provides an isolated nucleic acid encoding kidney alpha kinase, or a fragment thereof having at least 15 nucleotides. In particular, the invention includes an isolated nucleic acid encoding human kidney alpha kinase,

30 wherein the nucleic acid is selected from the group consisting of :

- a. the DNA sequence of SEQ ID NO: 30;



- b. DNA sequences that hybridize to the sequence of subpart (a) under moderate stringency hybridization conditions;
- c. DNA sequences capable of encoding the amino acid sequence encoded by the DNA sequences of (a) or (b);
- 5 d. degenerate variants thereof;
- e. alleles thereof; and
- f. hybridizable fragments thereof.

In particular, the invention includes an isolated nucleic acid encoding mouse kidney

10 alpha kinase, wherein the nucleic acid is selected from the group consisting of :

- a. the DNA sequence of SEQ ID NO: 32;
- b. DNA sequences that hybridize to the sequence of subpart (a) under moderate stringency hybridization conditions;
- c. DNA sequences capable of encoding the amino acid sequence encoded
- 15 by the DNA sequences of (a) or (b);
- d. degenerate variants thereof;
- e. alleles thereof; and
- f. hybridizable fragments thereof.

20 In particular, the invention provides an isolated nucleic acid encoding mammalian kidney alpha kinase, wherein the nucleic acid is selected from the group consisting of :

- a. the DNA sequence of SEQ ID NO: 30;
- b. the DNA sequence of SEQ ID NO: 32;
- 25 c. DNA sequences that hybridize to the sequence of subparts (a) or (b) under standard hybridization conditions; and
- d. DNA sequences capable of encoding the amino acid sequence encoded by the DNA sequences of subparts (a), (b) or (c).

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The present invention also provides an isolated nucleic acid encoding skeletal muscle alpha kinase, or a fragment thereof having at least 15 nucleotides. In particular, an isolated nucleic acid encoding skeletal muscle alpha kinase is provided, wherein the nucleic acid is selected from the group consisting of :

- 5           a.       nucleic acid comprising the DNA sequence of SEQ ID NO: 38;
- b.       DNA sequences that hybridize to the sequence of subpart (a) under moderate stringency hybridization conditions;
- c.       DNA sequences capable of encoding the amino acid sequence encoded by the DNA sequences of (a) or (b);
- 10          d.       degenerate variants thereof;
- e.       alleles thereof; and
- f.       hybridizable fragments thereof.

In particular, the invention provides an isolated nucleic acid encoding mammalian  
15 skeletal muscle alpha kinase, wherein the nucleic acid is selected from the group consisting of :

- a.       the DNA sequence of SEQ ID NO: 38;
- b.       DNA sequences that hybridize to the sequence of subpart (a) under standard hybridization conditions; and
- 20          c.       DNA sequences capable of encoding the amino acid sequence encoded by the DNA sequences of (a) or (b).

The present invention also includes an isolated nucleic acid encoding lymphocyte alpha kinase, or a fragment thereof having at least 15 nucleotides. In particular, the  
25 present invention provides an isolated nucleic acid encoding lymphocyte alpha kinase, wherein the nucleic acid is selected from the group consisting of :

- a.       nucleic acid comprising the DNA sequence of SEQ ID NO: 40;
- b.       DNA sequences that hybridize to the sequence of subpart (a) under moderate stringency hybridization conditions;
- 30          c.       DNA sequences capable of encoding the amino acid sequence encoded by the DNA sequences of (a) or (b);

- d. degenerate variants thereof;
- e. alleles thereof; and
- f. hybridizable fragments thereof.

5 In particular, the invention provides an isolated nucleic acid encoding mammalian lymphocyte alpha kinase, wherein the nucleic acid is selected from the group consisting of :

- a. the DNA sequence of SEQ ID NO: 40;
- b. DNA sequences that hybridize to the sequence of subpart (a) under  
10 standard hybridization conditions; and
- c. DNA sequences capable of encoding the amino acid sequence encoded by the DNA sequences of (a) or (b).

The invention provides an isolated nucleic acid encoding human melanoma alpha  
15 kinase, wherein the nucleic acid comprises the DNA sequence of SEQ ID NO: 26.

The invention provides an isolated nucleic acid encoding mouse melanoma alpha kinase, wherein the nucleic acid comprises the DNA sequence of SEQ ID NO: 28.

The invention provides an isolated nucleic acid encoding human heart alpha kinase,  
20 wherein the nucleic acid comprises the DNA sequence of SEQ ID NO: 34. The invention provides an isolated nucleic acid encoding mouse heart alpha kinase, wherein the nucleic acid comprises the DNA sequence of SEQ ID NO: 36.

The invention provides an isolated nucleic acid encoding human kidney alpha kinase,  
25 wherein the nucleic acid comprises the DNA sequence of SEQ ID NO: 30. The invention provides an isolated nucleic acid encoding mouse kidney alpha kinase, wherein the nucleic acid comprises the DNA sequence of SEQ ID NO: 32.

The invention provides an isolated nucleic acid encoding human skeletal muscle alpha  
30 kinase, wherein the nucleic acid comprises the DNA sequence of SEQ ID NO: 38.

The invention provides an isolated nucleic acid encoding human lymphocyte alpha kinase, wherein the nucleic acid comprises the DNA sequence of SEQ ID NO: 40.

5 The present invention also relates to a recombinant DNA molecule or cloned gene, or a degenerate variant thereof, which encodes an alpha kinase selected from the group of melanoma kinase, heart kinase, kidney kinase, skeletal muscle kinase and lymphocyte kinase; preferably a nucleic acid molecule, in particular a recombinant DNA molecule or cloned gene, encoding the alpha kinase has a nucleotide sequence or is complementary to a DNA sequence as set forth in any of SEQ ID NOS: 26, 28,  
10 30, 32, 34, 36, 38 and 40.

The murine and/or human DNA sequences of the alpha kinase genes of the present invention or portions thereof, may be prepared as probes to screen for complementary sequences and genomic clones in the same or alternate species. The present invention  
15 extends to probes so prepared that may be provided for screening cDNA and genomic libraries for the alpha kinase genes. For example, the probes may be prepared with a variety of known vectors, such as the phage  $\lambda$  vector. The present invention also includes the preparation of plasmids including such vectors, and the use of the DNA sequences to construct vectors expressing antisense RNA or ribozymes which would  
20 attack the mRNAs of any or all of the DNA sequences set forth in any of SEQ ID NOS: 26, 28, 30, 32, 34, 36, 38 and 40. Correspondingly, the preparation of antisense RNA and ribozymes are included herein.

According to other preferred features of certain preferred embodiments of the present  
25 invention, a recombinant expression system is provided to produce biologically active animal or human alpha kinase selected from the group of melanoma kinase, heart kinase, kidney kinase, skeletal muscle kinase and lymphocyte kinase.

The present invention naturally contemplates several means for preparation of the  
30 alpha kinase of the present invention, including as illustrated herein known

recombinant techniques, and the invention is accordingly intended to cover such synthetic preparations within its scope. The isolation of the cDNA and amino acid sequences disclosed herein facilitates the production of the alpha kinase of the present invention by such recombinant techniques, and accordingly, the invention extends to  
 5 expression vectors prepared from the disclosed DNA sequences for expression in host systems by recombinant DNA techniques, and to the resulting transformed hosts.

In a further aspect, the invention provides a recombinant DNA expression vector comprising the nucleic acid encoding an alpha kinase protein selected from the group  
 10 of melanoma alpha kinase, kidney alpha kinase, heart alpha kinase, skeletal muscle alpha kinase and lymphocyte alpha kinase, wherein the DNA encoding the alpha kinase is operatively associated with an expression control sequence. The invention also provides a transformed host cell transfected with said DNA vector.

- 15 The invention further includes a unicellular host transformed with a recombinant DNA molecule comprising a DNA sequence or degenerate variant thereof, which encodes an alpha kinase, or a fragment thereof, selected from the group consisting of:
- a. the DNA sequence of (SEQ ID NO: 26);
  - b. the DNA sequence of (SEQ ID NO: 28);
  - 20 c. the DNA sequence of (SEQ ID NO: 30);
  - d. the DNA sequence of (SEQ ID NO: 32);
  - e. the DNA sequence of (SEQ ID NO: 34);
  - f. the DNA sequence of (SEQ ID NO: 36);
  - g. the DNA sequence of (SEQ ID NO: 38);
  - 25 h. the DNA sequence of (SEQ ID NO: 40);
  - i. DNA sequences that hybridize to any of the foregoing DNA sequences under standard hybridization conditions; and
  - j. DNA sequences that code on expression for an amino acid sequence encoded by any of the foregoing DNA sequences;
  - 30 wherein said DNA sequence is operatively linked to an expression control sequence.

Such a unicellular host is particularly selected from the group consisting of *E. coli*, *Pseudomonas*, *Bacillus*, *Streptomyces*, yeasts, CHO, R1.1, B-W, L-M, COS 1, COS 7, BSC1, BSC40, and BMT10 cells, plant cells, insect cells, mouse cells and human cells in tissue culture.

In a further aspect, the present invention includes an isolated protein characterized by the presence of at least two domains, one of the domains being an alpha-kinase catalytic domain and the other domain being an ion channel domain.

Thus, the present invention provides an isolated melanoma alpha kinase protein characterized by having an alpha-kinase catalytic domain and an ion channel domain. In particular, a melanoma alpha kinase protein is provided which comprises the amino acid sequence set out in SEQ ID NO: 27 and 29, and analogs, variants and fragments thereof. The invention provides a melanoma alpha kinase protein which comprises the amino acid sequence set out in SEQ ID NO: 27 or 29, and variants thereof wherein one or more amino acids is substituted with a conserved amino acid.

The invention further provides an isolated kidney alpha kinase protein characterized by having an alpha-kinase catalytic domain and an ion channel domain. In particular, the kidney alpha kinase protein comprises the amino acid sequence set out in SEQ ID NO: 31 and 33, and analogs, variants and fragments thereof. The invention provides a kidney alpha kinase protein which comprises the amino acid sequence set out in SEQ ID NO: 31 or 33, and variants thereof wherein one or more amino acids is substituted with a conserved amino acid.

The present invention further provides an isolated heart alpha kinase protein. In particular, the heart alpha kinase protein comprises the amino acid sequence set out in SEQ ID NO: 35 and 37, and analogs, variants and immunogenic fragments thereof. The invention provides a heart alpha kinase protein which comprises the amino acid sequence set out in SEQ ID NO: 35 or 37, and variants thereof wherein one or more amino acids is substituted with a conserved amino acid.

The present invention still further provides an isolated skeletal muscle alpha kinase protein. In particular, the skeletal muscle alpha kinase protein comprises the amino acid sequence set out in SEQ ID NO: 39, and analogs, variants and immunogenic fragments thereof. The invention provides a skeletal muscle alpha kinase protein which comprises the amino acid sequence set out in SEQ ID NO: 39, and variants thereof wherein one or more amino acids is substituted with a conserved amino acid.

The invention includes an isolated lymphocyte alpha kinase protein. In particular, the lymphocyte alpha kinase protein comprises the amino acid sequence set out in SEQ ID NO: 41, and analogs, variants and immunogenic fragments thereof. The invention provides a lymphocyte alpha kinase protein which comprises the amino acid sequence set out in SEQ ID NO: 41, and variants thereof wherein one or more amino acids is substituted with a conserved amino acid.

In a particular aspect, the present invention includes a pharmaceutical composition comprising one or more alpha kinase protein selected from the group of melanoma alpha kinase, kidney alpha kinase, heart alpha kinase, skeletal muscle alpha kinase and lymphocyte alpha kinase, and a pharmaceutically acceptable carrier.

In a further aspect, the invention provides a purified antibody to an alpha kinase protein selected from the group of melanoma alpha kinase, kidney alpha kinase, heart alpha kinase, skeletal muscle alpha kinase and lymphocyte alpha kinase.

A monoclonal antibody to an alpha kinase protein selected from the group of melanoma alpha kinase, kidney alpha kinase, heart alpha kinase, skeletal muscle alpha kinase and lymphocyte alpha kinase is still further provided. the invention includes an immortal cell line that produces a monoclonal antibody to an alpha kinase protein selected from the group of melanoma alpha kinase, kidney alpha kinase, heart alpha kinase, skeletal muscle alpha kinase and lymphocyte alpha kinase.

Any such contemplated antibody may be labeled with a detectable label. The label may be selected from the group consisting of an enzyme, a chemical which fluoresces, and a radioactive element.

The invention further includes an antibody to an alpha kinase protein selected from the group of melanoma alpha kinase, kidney alpha kinase, heart alpha kinase, skeletal muscle alpha kinase and lymphocyte alpha kinase, which recognizes the phosphorylated form of the alpha kinase or a phosphorylated fragment thereof.

The present invention likewise extends to antibodies against specifically phosphorylated alpha kinase targets, including naturally raised and recombinantly prepared antibodies. These antibodies and their labeled counterparts are included within the scope of the present invention for their particular ability in detecting alpha kinase activity *via* detection of the phosphorylated product by ELISA or any other immunoassay known to the skilled artisan.

In the instance where a radioactive label, such as the isotopes  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{33}\text{P}$ ,  $^{35}\text{S}$ ,  $^{36}\text{Cl}$ ,  $^{51}\text{Cr}$ ,  $^{57}\text{Co}$ ,  $^{58}\text{Co}$ ,  $^{59}\text{Fe}$ ,  $^{90}\text{Y}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ , and  $^{186}\text{Re}$  are used, known currently available counting procedures may be utilized. In the instance where the label is an enzyme, detection may be accomplished by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques known in the art.

The present invention provides a method for treating an animal in need of increased activity of melanoma alpha kinase which comprises administration of melanoma alpha kinase to the animal.

The present invention further provides a method for treating an animal in need of increased activity of melanoma alpha kinase which comprises administration of an antibody against melanoma alpha kinase to the animal.



The present invention also provides a method for treating an animal in need of increased activity of kidney alpha kinase which comprises administration of kidney alpha kinase to the animal.

The invention also includes a method for treating an animal in need of increased activity of kidney alpha kinase which comprises administration of an antibody against kidney alpha kinase to the animal.

The invention further provides a method for treating an animal in need of increased activity of heart alpha kinase which comprises administration of heart alpha kinase to the animal.

The present invention also contemplates a method for treating an animal in need of increased activity of heart alpha kinase which comprises administration of an antibody against heart alpha kinase to the animal.

In an additional aspect, the invention provides a method for treating an animal in need of increased activity of skeletal muscle alpha kinase which comprises administration of skeletal muscle alpha kinase to the animal.

A method for treating an animal in need of increased activity of skeletal muscle alpha kinase which comprises administration of an antibody against skeletal muscle alpha kinase to the animal is further provided.

The present invention includes method for treating an animal in need of increased activity of lymphocyte alpha kinase which comprises administration of lymphocyte alpha kinase to the animal.

The present invention further provides a method for treating an animal in need of increased activity of lymphocyte alpha kinase which comprises administration of an antibody against lymphocyte alpha kinase to the animal.

The therapeutic method provided herein could include the method for the treatment of various pathologies or other cellular dysfunctions and derangements by the administration of pharmaceutical compositions that may comprise effective inhibitors of alpha kinase activity, or other equally effective drugs developed for instance by a drug screening assay prepared and used in accordance with a further aspect of the present invention.

The invention includes an assay system for screening of potential drugs effective at attenuating alpha kinase activity of target mammalian cells by interrupting or potentiating the phosphorylation of alpha kinase selected from the group of melanoma kinase, heart kinase, kidney kinase, skeletal muscle kinase and lymphocyte kinase. In one instance, the test drug could be administered to a cellular sample along with ATP carrying a detectable label on its  $\gamma$ -phosphate that gets transferred to the kinase target, including the kinase itself, or a peptide substrate, by the particular alpha kinase. Quantification of the labeled kinase target or peptide substrate is diagnostic of the candidate drug's efficacy. A further embodiment would provide for the assay to be performed using a purely *in vitro* system comprised of the alpha kinase, ATP or labeled ATP, the kinase target or peptide substrate, appropriate buffer, and detection reagents and/or instrumentation to detect and quantify the extent of alpha kinase-directed phosphorylation activity.

The assay system could more importantly be adapted to identify drugs or other entities that are capable of binding to the alpha kinase and/or its cognate phosphorylation target, either in the cytoplasm or in the nucleus, thereby inhibiting or potentiating alpha kinase activity and its resultant phenotypic outcome. Such an assay would be useful in the development of drugs that would be specific against particular cellular activity, or that would potentiate such activity, in time or in level of activity. For example, such drugs might be used to treat various carcinomas or other hyperproliferative pathologies.

In an additional aspect, the present invention includes a method for detecting the presence or activity of an alpha kinase protein selected from the group of melanoma alpha kinase, kidney alpha kinase, heart alpha kinase, skeletal muscle alpha kinase and lymphocyte alpha kinase, wherein said alpha kinase is measured by:

A. contacting a biological sample from a mammal in which the presence or activity of said alpha kinase is suspected with a binding partner of said alpha kinase under conditions that allow binding of said alpha kinase to said binding partner to occur; and

B. detecting whether binding has occurred between said alpha kinase from said sample and the binding partner;

wherein the detection of binding indicates that presence or activity of said alpha kinase in said sample.

The present invention further provides a method for detecting the presence of an alpha kinase protein selected from the group of melanoma alpha kinase, kidney alpha kinase, heart alpha kinase, skeletal muscle alpha kinase and lymphocyte alpha kinase, wherein the alpha kinase is measured by:

a. contacting a sample in which the presence or activity of an alpha kinase protein selected from the group of melanoma alpha kinase, kidney alpha kinase, heart alpha kinase, skeletal muscle alpha kinase and lymphocyte alpha kinase is suspected with an antibody to the said alpha kinase protein under conditions that allow binding of the alpha kinase protein to the binding partner to occur; and

b. detecting whether binding has occurred between the alpha kinase protein from the sample and the antibody;

wherein the detection of binding indicates the presence or activity of the alpha kinase protein in the sample.

In a still further aspect, the invention provides a method of testing the ability of a drug or other entity to modulate the kinase activity of an alpha kinase protein selected from

the group of melanoma alpha kinase, kidney alpha kinase, heart alpha kinase, skeletal muscle alpha kinase and lymphocyte alpha kinase which comprises:

- A. culturing a colony of test cells containing the alpha kinase protein;
- B. adding the drug or other entity under test; and
- C. measuring the kinase activity of said alpha kinase protein in the test

cells, wherein when the amount of kinase activity in the presence of the modulator is greater than in its absence, the modulator is identified as an agonist or activator of the alpha kinase protein, whereas when the amount of kinase activity in the presence of the modulator is less than in its absence, the modulator is identified as an antagonist or inhibitor of the alpha kinase protein.

Other objects and advantages will become apparent to those skilled in the art from a review of the ensuing description which proceeds with reference to the following illustrative drawings.

#### BRIEF DESCRIPTION OF THE DRAWINGS

**FIGURE 1.** A, Sequence alignment of the catalytic domains of human eEF-2 kinase, *C. elegans* eEF-2 kinase, MHCK A, MHCK B and clone FC-ANO9. Identical amino acids (bold) and conserved hydrophobic amino acids (°) are noted. B, Phylogenetic tree of sequences shown in (A), with the addition of mouse and rat eEF-2 kinases. Tree was obtained using the J. Hein method with PAM250 residue weight table. The following accession numbers were used for the sequences: U93846-U93850, 1495779, 1170675, 1903458, C22986.

**FIGURE 2** depicts a sequence alignment of *C. elegans*, mouse, human eEF-2 kinase, and the catalytic domain of *Dictyostelium discoideum* MHCK A. Identical amino acids are indicated by dark blue boxed regions and chemically conserved amino acids are indicated by light blue shaded regions. Amino acids in the human sequence that are identical to the mouse sequence are represented by dots. Amino acids underlined

in black correspond to the six regions that match peptides obtained from the sequencing of purified rabbit reticulocyte eEF-2 kinase. The GXGXXG nucleotide-binding motif is underlined in red. The blue dashed line over residues 625-632 in *C. elegans* eEF-2 kinases designates the amino acids corresponding to exon 4, which is missing in *Cefk-2*.

**FIGURE 3** depicts a schematic representation of the structure of mammalian and *C. elegans* eEF-2 kinases and MHCK A. The homologous regions are represented by dark shading. The regions of weak similarity are represented by light shading. The position of the GXGXXG motif is indicated by vertical arrows.

**FIGURE 4** depicts a sequence alignment of *C. elegans*, mouse, human eEF-2 kinase, and the catalytic domain of *Dictyostelium discoideum* MHCK A, heart kinase, melanoma kinase and ch4 kinase. Identical amino acids are indicated by dark blue boxed regions and chemically conserved amino acids are indicated by light blue shaded regions.

**FIGURE 5 A-C** depicts the nucleic acid sequence of mouse melanoma alpha-kinase (MK).

**FIGURE 6 A-B** depicts the predicted amino acid sequence of mouse melanoma alpha-kinase (MK).

**FIGURE 7 A and B** depicts the nucleic acid sequence (A) and predicted amino acid sequence (B) of human melanoma alpha-kinase (MK).

**FIGURE 8 A and B** depicts the nucleic acid sequence (A) and predicted amino acid sequence (B) of human heart alpha-kinase (HK).

**FIGURE 9 A and B** depicts the nucleic acid sequence (A) and predicted amino acid sequence (B) of human kidney alpha-kinase (KK).

**FIGURE 10 A and B** depicts the nucleic acid sequence (A) and predicted amino acid sequence (B) of human skeletal muscle alpha-kinase (SK).

**FIGURE 11 A and B** depicts the nucleic acid sequence (A) and predicted amino acid sequence (B) of human lymphocyte alpha-kinase (LK).

**FIGURE 12** shows the alignment of the catalytic domains of the cloned alpha-kinases.

**FIGURE 13** depicts a phylogeneic analysis of the cloned alpha-kinases.

**FIGURE 14** shows the time course of  $^{32}\text{P}$  incorporation into expressed maltose-binding protein-melanoma alpha-kinase fusion protein (MBP-MK).

**FIGURE 15** shows Northern Blot analysis of the tissue distribution of the alpha-kinases in human and mouse tissues. Standard Multiple Tissue Northern (MTN) blots (Clontech) were stained as described in Materials and Methods. A, B, C: Blots probed for Melanoma Kinase; A: Human MTN Blot B: Human Immune System MTN Blot II. C: Mouse MTN Blot. D: Human 12-Lane MTN Blot probed for Kidney kinase. E: Human 12-Lane MTN Blot probed for Muscle kinase. F: Mouse MTN Blot probed for Heart kinase. (abbreviations: sk. muscle - skeletal muscle, p.b. leukocyte - peripheral blood leukocyte, s. intestine - small intestine).

**FIGURE 16** shows a comparison of the ion channel portions of melanoma kinase (MK), kidney kinase (KK) and melastatin (ME).

**FIGURE 17** Sequence alignment of MK and KK with members of the LTRP channel subfamily. Roman numerals designate the six predicted transmembrane segments. Black boxes highlight identical amino acids. Gray boxes highlight conserved amino acids. The alignment was constructed using the ClustalW program and the shading was done using the Boxshade program.

**FIGURE 18.** Schematic representation of five new  $\alpha$ -kinases described in this paper together with eEF-2 kinase and the *Dictyostelium* MHCKs.

**FIGURE 19. A.** Phylogenetic tree of the LTRP channel subfamily. This tree was generated from the full-length protein sequences using the ClustalW program. **B.** The proposed structural model of MK and KK.

### DETAILED DESCRIPTION

Protein phosphorylation plays a pivotal role in a wide variety of cellular processes. Enzymes which assist in protein phosphorylation are referred to as “protein kinases.” Two protein kinase superfamilies have been described. The vast majority of protein kinases belong to the serine/threonine/tyrosine kinase superfamily. Several hundred members of this superfamily have thus far been characterized and found to share similar structural organization of their catalytic domains consisting of 12 conserved subdomains. There is also the histidine kinase superfamily consisting primarily of sensor components of the prokaryotic two-component signal transduction systems. Eukaryotic members of this superfamily have been recently described. In addition, mitochondrial branched-chain  $\alpha$ -ketoacid dehydrogenase kinase and the mitochondrial pyruvate dehydrogenase kinase have been described which are structurally related to the histidine kinases, but phosphorylate their substrates on serine. The existence of several protein kinases have recently been reported which have very little or no homology to either superfamily. This new superfamily is termed alpha-kinase. The first two members eEF-2 kinase and MHCKA kinase differ from conventional protein

kinases in that they phosphorylate amino acids located within  $\alpha$ -helices. Thus, in addition to the two well-characterized superfamily of eukaryotic protein kinases, which phosphorylate amino acids located in loops and turns, there appears to be a third superfamily of  $\alpha$ -helix-directed kinases.

Additional novel members of the alpha kinase superfamily have herein been cloned and sequenced. In particular, these new alpha kinases - melanoma alpha kinase, kidney alpha kinase, heart alpha kinase, skeletal muscle alpha kinase and lymphocyte kinase - represent new members of the alpha kinase superfamily. The alpha kinases of the present invention are related to eEF-2 kinase and MHCK A and join the alpha kinase superfamily. In addition, however, the novel alpha kinases of the present invention have new and unique characteristics.

In particular, the melanoma alpha kinase and kidney kinase of the present invention have a unique structure. These proteins have two domains, one domain is the alpha-kinase catalytic domain and the other is an ion channel. This is the first recognized example of an ion channel being covalently linked to a protein kinase. It is likely that these novel protein kinases can be regulated by ion flow through the membrane.

Expression of the melanoma kinase was detected in all mouse tissues studied, including heart, skeletal muscle, brain, liver and lung. This kinase is the most abundant in the heart. In contrast, the kidney kinase is present almost exclusively in kidney tissue. The ion channel portion is very similar to (70% identical) to a previously identified protein called melastatin that is selectively downregulated in metastatic tumors, and therefore is believed to be a metastasis suppressor gene.

Melanoma alpha-kinase, kidney alpha-kinase, as well as melastatin, belong to the TRP family of ion channels. All TRP proteins function as tetramers, and various TRP proteins can form tetramers in different combinations that result in ion channels with different properties. Considering the high degree of similarity between melanoma kinase, kidney kinase, and melastatin, it is likely that melanoma kinase and kidney kinase can form tetrameric complexes with melastatin.



In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook et al, "Molecular Cloning: A Laboratory Manual" (1989); "Current Protocols in Molecular Biology" Volumes I-III [Ausubel, R. M., ed. (1994)]; "Cell Biology: A Laboratory Handbook" Volumes I-III [J. E. Celis, ed. (1994)]; "Current Protocols in Immunology" Volumes I-III [Coligan, J. E., ed. (1994)]; "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" [B.D. Hames & S.J. Higgins eds. (1985)]; "Transcription And Translation" [B.D. Hames & S.J. Higgins, eds. (1984)]; "Animal Cell Culture" [R.I. Freshney, ed. (1986)]; "Immobilized Cells And Enzymes" [IRL Press, (1986)]; B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

Therefore, if appearing herein, the following terms shall have the definitions set out below.

The terms "elongation factor-2 kinase", "eEF-2 kinase", "EF-2 kinase", "Cefk", and any variants not specifically listed, may be used herein interchangeably, and as used throughout the present application and claims refer to proteinaceous material including single or multiple proteins, and extends to those proteins having the amino acid sequence data described herein and presented in FIGURES 1 and 5 (SEQ ID NOS: 1, 2, 6, 8 and 14), and the profile of activities set forth herein and in the Claims. Accordingly, proteins displaying substantially equivalent or altered activity are likewise contemplated. These modifications may be deliberate, for example, such as modifications obtained through site-directed mutagenesis, or may be accidental, such as those obtained through mutations in hosts that are producers of the complex or its named subunits. Also, the terms "elongation factor-2 kinase", "eEF-2 kinase", "EF-2 kinase", and "Cefk" are intended to include within their scope proteins specifically recited herein as well as all substantially homologous analogs and allelic variations.

The terms "melanoma  $\alpha$  kinase", "melanoma alpha kinase ", "melanoma kinase", "MK", and any variants not specifically listed, may be used herein interchangeably, and as used throughout the present application and claims refer to proteinaceous material including single or multiple proteins, and extends to those proteins having the amino acid sequence data described herein and presented in FIGURES 6 and 7 (SEQ ID NOS: 27 and 29), and the profile of activities set forth herein and in the Claims. Accordingly, proteins displaying substantially equivalent or altered activity are likewise contemplated. These modifications may be deliberate, for example, such as modifications obtained through site-directed mutagenesis, or may be accidental, such as those obtained through mutations in hosts that are producers of the complex or its named subunits. Also, the terms "melanoma  $\alpha$  kinase", "melanoma alpha kinase ", "melanoma kinase" and "MK" are intended to include within their scope proteins specifically recited herein as well as all substantially homologous analogs and allelic variations.

The terms "heart  $\alpha$  kinase", "heart alpha kinase ", "heart kinase", "HK", and any variants not specifically listed, may be used herein interchangeably, and as used throughout the present application and claims refer to proteinaceous material including single or multiple proteins, and extends to those proteins having the amino acid sequence data described herein and presented in FIGURE 8 (SEQ ID NOS: 35 and 37), and the profile of activities set forth herein and in the Claims. Accordingly, proteins displaying substantially equivalent or altered activity are likewise contemplated. These modifications may be deliberate, for example, such as modifications obtained through site-directed mutagenesis, or may be accidental, such as those obtained through mutations in hosts that are producers of the complex or its named subunits. Also, the terms "heart  $\alpha$  kinase", "heart alpha kinase ", "heart kinase", "HK" are intended to include within their scope proteins specifically recited herein as well as all substantially homologous analogs and allelic variations.

The terms "kidney  $\alpha$  kinase", "kidney alpha kinase ", "kidney kinase", "KK", and any variants not specifically listed, may be used herein interchangeably, and as used throughout the present application and claims refer to proteinaceous material including single or multiple proteins, and extends to those proteins having the amino acid sequence data described herein and presented in FIGURE 9 (SEQ ID NOS: 31 and 33), and the profile of activities set forth herein and in the Claims. Accordingly, proteins displaying substantially equivalent or altered activity are likewise contemplated. These modifications may be deliberate, for example, such as modifications obtained through site-directed mutagenesis, or may be accidental, such as those obtained through mutations in hosts that are producers of the complex or its named subunits. Also, the terms "kidney  $\alpha$  kinase", "kidney alpha kinase ", "kidney kinase", "KK" are intended to include within their scope proteins specifically recited herein as well as all substantially homologous analogs and allelic variations.

The terms "skeletal muscle  $\alpha$  kinase", "skeletal muscle alpha kinase ", "skeletal muscle kinase", "SK", and any variants not specifically listed, may be used herein interchangeably, and as used throughout the present application and claims refer to proteinaceous material including single or multiple proteins, and extends to those proteins having the amino acid sequence data described herein and presented in FIGURE 10 (SEQ ID NO: 39), and the profile of activities set forth herein and in the Claims. Accordingly, proteins displaying substantially equivalent or altered activity are likewise contemplated. These modifications may be deliberate, for example, such as modifications obtained through site-directed mutagenesis, or may be accidental, such as those obtained through mutations in hosts that are producers of the complex or its named subunits. Also, the terms "skeletal muscle  $\alpha$  kinase", "skeletal muscle alpha kinase ", "skeletal muscle kinase", "SK" are intended to include within their scope proteins specifically recited herein as well as all substantially homologous analogs and allelic variations.

The terms "lymphocyte  $\alpha$  kinase", "lymphocyte alpha kinase ", "lymphocyte kinase", "LK", "Ch4" and any variants not specifically listed, may be used herein interchangeably, and as used throughout the present application and claims refer to proteinaceous material including single or multiple proteins, and extends to those proteins having the amino acid sequence data described herein and presented in FIGURE 11 (SEQ ID NO: 41), and the profile of activities set forth herein and in the Claims. Accordingly, proteins displaying substantially equivalent or altered activity are likewise contemplated. These modifications may be deliberate, for example, such as modifications obtained through site-directed mutagenesis, or may be accidental, such as those obtained through mutations in hosts that are producers of the complex or its named subunits. Also, the terms "lymphocyte  $\alpha$  kinase", "lymphocyte alpha kinase ", "lymphocyte kinase", "LK", "Ch4" are intended to include within their scope proteins specifically recited herein as well as all substantially homologous analogs and allelic variations.

The amino acid residues described herein are preferred to be in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired fractional property of immunoglobulin-binding is retained by the polypeptide.  $\text{NH}_2$  refers to the free amino group present at the amino terminus of a polypeptide.  $\text{COOH}$  refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature, *J. Biol. Chem.*, **243**:3552-59 (1969), abbreviations for amino acid residues are shown in the following Table of Correspondence:

TABLE OF CORRESPONDENCE

<u>SYMBOL</u>		<u>AMINO ACID</u>
<u>1-Letter</u>	<u>3-Letter</u>	
Y	Tyr	tyrosine
G	Gly	glycine
F	Phe	phenylalanine

M	Met	methionine
A	Ala	alanine
S	Ser	serine
I	Ile	isoleucine
L	Leu	leucine
T	Thr	threonine
V	Val	valine
P	Pro	proline
K	Lys	lysine
H	His	histidine
Q	Gln	glutamine
E	Glu	glutamic acid
W	Trp	tryptophan
R	Arg	arginine
D	Asp	aspartic acid
N	Asn	asparagine
C	Cys	cysteine

It should be noted that all amino-acid residue sequences are represented herein by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino-acid residues. The above Table is presented to correlate the three-letter and one-letter notations which may appear alternately herein.

### Nucleic Acids

The present invention provides an isolated nucleic acid encoding melanoma alpha kinase, or a fragment thereof having at least 15 nucleotides. In particular, the

invention provides an isolated nucleic acid encoding human melanoma alpha kinase, wherein the nucleic acid is selected from the group consisting of :

- a. the DNA sequence of SEQ ID NO: 26;
- b. DNA sequences that hybridize to the sequence of subpart (a) under moderate stringency hybridization conditions;
- c. DNA sequences capable of encoding the amino acid sequence encoded by the DNA sequences of (a) or (b);
- d. degenerate variants thereof;
- e. alleles thereof; and
- f. hybridizable fragments thereof.

In particular, the invention provides an isolated nucleic acid encoding mouse melanoma alpha kinase, wherein the nucleic acid is selected from the group consisting of :

- a. the DNA sequence of SEQ ID NO: 28;
- b. DNA sequences that hybridize to the sequence of subpart (a) under moderate stringency hybridization conditions;
- c. DNA sequences capable of encoding the amino acid sequence encoded by the DNA sequences of (a) or (b);
- d. degenerate variants thereof;
- e. alleles thereof; and
- f. hybridizable fragments thereof.

The present invention further provides an isolated nucleic acid encoding heart alpha kinase, or a fragment thereof having at least 15 nucleotides. In particular, the present invention provides an isolated nucleic acid encoding human heart alpha kinase, wherein the nucleic acid is selected from the group consisting of :

- a. nucleic acid comprising the DNA sequence of SEQ ID NO: 34;
- b. DNA sequences that hybridize to the sequence of subpart (a) under moderate stringency hybridization conditions;

- c. DNA sequences capable of encoding the amino acid sequence encoded by the DNA sequences of (a) or (b);
- d. degenerate variants thereof;
- e. alleles thereof; and
- f. hybridizable fragments thereof.

In particular, the present invention provides an isolated nucleic acid encoding mouse heart alpha kinase, wherein the nucleic acid is selected from the group consisting of :

- a. nucleic acid comprising the DNA sequence of SEQ ID NO: 36;
- b. DNA sequences that hybridize to the sequence of subpart (a) under moderate stringency hybridization conditions;
- c. DNA sequences capable of encoding the amino acid sequence encoded by the DNA sequences of (a) or (b);
- d. degenerate variants thereof;
- e. alleles thereof; and
- f. hybridizable fragments thereof.

The present invention still further provides an isolated nucleic acid encoding kidney alpha kinase, or a fragment thereof having at least 15 nucleotides. In particular, the invention includes an isolated nucleic acid encoding human kidney alpha kinase, wherein the nucleic acid is selected from the group consisting of :

- a. the DNA sequence of SEQ ID NO: 30;
- b. DNA sequences that hybridize to the sequence of subpart (a) under moderate stringency hybridization conditions;
- c. DNA sequences capable of encoding the amino acid sequence encoded by the DNA sequences of (a) or (b);
- d. degenerate variants thereof;
- e. alleles thereof; and
- f. hybridizable fragments thereof.

In particular, the invention includes an isolated nucleic acid encoding mouse kidney alpha kinase, wherein the nucleic acid is selected from the group consisting of :

- a. the DNA sequence of SEQ ID NO: 32;
- b. DNA sequences that hybridize to the sequence of subpart (a) under moderate stringency hybridization conditions;
- c. DNA sequences capable of encoding the amino acid sequence encoded by the DNA sequences of (a) or (b);
- d. degenerate variants thereof;
- e. alleles thereof; and
- f. hybridizable fragments thereof.

The present invention also provides an isolated nucleic acid encoding skeletal muscle alpha kinase, or a fragment thereof having at least 15 nucleotides. In particular, an isolated nucleic acid encoding skeletal muscle alpha kinase is provided, wherein the nucleic acid is selected from the group consisting of :

- a. nucleic acid comprising the DNA sequence of SEQ ID NO: 38;
- b. DNA sequences that hybridize to the sequence of subpart (a) under moderate stringency hybridization conditions;
- c. DNA sequences capable of encoding the amino acid sequence encoded by the DNA sequences of (a) or (b);
- d. degenerate variants thereof;
- e. alleles thereof; and
- f. hybridizable fragments thereof.

The present invention also includes an isolated nucleic acid encoding lymphocyte alpha kinase, or a fragment thereof having at least 15 nucleotides. In particular, the present invention provides an isolated nucleic acid encoding lymphocyte alpha kinase, wherein the nucleic acid is selected from the group consisting of :

- a. nucleic acid comprising the DNA sequence of SEQ ID NO: 40;
- b. DNA sequences that hybridize to the sequence of subpart (a) under moderate stringency hybridization conditions;



- c. DNA sequences capable of encoding the amino acid sequence encoded by the DNA sequences of (a) or (b);
- d. degenerate variants thereof;
- e. alleles thereof; and
- f. hybridizable fragments thereof.

The present invention also relates to a recombinant DNA molecule or cloned gene, or a degenerate variant thereof, which encodes an alpha kinase selected from the group of melanoma kinase, heart kinase, kidney kinase, skeletal muscle kinase and lymphocyte kinase; preferably a nucleic acid molecule, in particular a recombinant DNA molecule or cloned gene, encoding the alpha kinase has a nucleotide sequence or is complementary to a DNA sequence as set forth in any of SEQ ID NOS: 26, 28, 30, 32, 34, 36, 38 and 40.

The murine and/or human DNA sequences of the alpha kinase genes of the present invention or portions thereof, may be prepared as probes to screen for complementary sequences and genomic clones in the same or alternate species. The present invention extends to probes so prepared that may be provided for screening cDNA and genomic libraries for the alpha kinase genes. For example, the probes may be prepared with a variety of known vectors, such as the phage  $\lambda$  vector. The present invention also includes the preparation of plasmids including such vectors, and the use of the DNA sequences to construct vectors expressing antisense RNA or ribozymes which would attack the mRNAs of any or all of the DNA sequences set forth in any of SEQ ID NOS: 26, 28, 30, 32, 34, 36, 38 and 40. Correspondingly, the preparation of antisense RNA and ribozymes are included herein.

According to other preferred features of certain preferred embodiments of the present invention, a recombinant expression system is provided to produce biologically active animal or human alpha kinase selected from the group of melanoma kinase, heart kinase, kidney kinase, skeletal muscle kinase and lymphocyte kinase.

The present invention naturally contemplates several means for preparation of the alpha kinase of the present invention, including as illustrated herein known recombinant techniques, and the invention is accordingly intended to cover such synthetic preparations within its scope. The isolation of the cDNA and amino acid sequences disclosed herein facilitates the production of the alpha kinase of the present invention by such recombinant techniques, and accordingly, the invention extends to expression vectors prepared from the disclosed DNA sequences for expression in host systems by recombinant DNA techniques, and to the resulting transformed hosts.

In a further aspect, the invention provides a recombinant DNA expression vector comprising the nucleic acid encoding an alpha kinase protein selected from the group of melanoma alpha kinase, kidney alpha kinase, heart alpha kinase, skeletal muscle alpha kinase and lymphocyte alpha kinase, wherein the DNA encoding the alpha kinase is operatively associated with an expression control sequence. The invention also provides a transformed host cell transfected with said DNA vector.

The invention further includes a unicellular host transformed with a recombinant DNA molecule comprising a DNA sequence or degenerate variant thereof, which encodes an alpha kinase, or a fragment thereof, selected from the group consisting of:

- a. the DNA sequence of (SEQ ID NO: 26);
- b. the DNA sequence of (SEQ ID NO: 28);
- c. the DNA sequence of (SEQ ID NO: 30);
- d. the DNA sequence of (SEQ ID NO: 32);
- e. the DNA sequence of (SEQ ID NO: 34);
- f. the DNA sequence of (SEQ ID NO: 36);
- g. the DNA sequence of (SEQ ID NO: 38);
- h. the DNA sequence of (SEQ ID NO: 40);
- i. DNA sequences that hybridize to any of the foregoing DNA sequences under standard hybridization conditions; and
- j. DNA sequences that code on expression for an amino acid sequence encoded by any of the foregoing DNA sequences;

wherein said DNA sequence is operatively linked to an expression control sequence.

Such a unicellular host is particularly selected from the group consisting of *E. coli*, *Pseudomonas*, *Bacillus*, *Streptomyces*, yeasts, CHO, R1.1, B-W, L-M, COS 1, COS 7, BSC1, BSC40, and BMT10 cells, plant cells, insect cells, mouse cells and human cells in tissue culture.

A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*; *i.e.*, capable of replication under its own control.

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA).

An "origin of replication" refers to those DNA sequences that participate in DNA synthesis.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a

start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

An "expression control sequence" is a DNA sequence that controls and regulates the transcription and translation of another DNA sequence. A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

A DNA sequence is "operatively linked" to an expression control sequence when the expression control sequence controls and regulates the transcription and translation of that DNA sequence. The term "operatively linked" includes having an appropriate start signal (e.g., ATG) in front of the DNA sequence to be expressed and maintaining the correct reading frame to permit expression of the DNA sequence under the control of the expression control sequence and production of the desired product encoded by the DNA sequence. If a gene that one desires to insert into a recombinant DNA molecule does not contain an appropriate start signal, such a start signal can be inserted in front of the gene.

The term "standard hybridization conditions" refers to salt and temperature conditions substantially equivalent to 5 x SSC and 65°C for both hybridization and wash. However, one skilled in the art will appreciate that such "standard hybridization conditions" are dependent on particular conditions including the concentration of sodium and magnesium in the buffer, nucleotide sequence length and concentration, percent mismatch, percent formamide, and the like. Also important in the determination of "standard hybridization conditions" is whether the two sequences hybridizing are RNA-RNA, DNA-DNA or RNA-DNA. Such standard hybridization conditions are easily determined by one skilled in the art according to well known formulae, wherein hybridization is typically 10-20°C below the predicted or determined  $T_m$  with washes of higher stringency, if desired.

In one aspect, the present invention relates to the identification of a new superfamily of protein kinases, denoted alpha kinases. Accordingly, it includes the DNA sequences coding for these family members. In addition, the invention also contemplates that each member of this new protein kinase superfamily has its own cognate phosphorylation target.

A "signal sequence" can be included before the coding sequence. This sequence encodes a signal peptide, N-terminal to the polypeptide, that communicates to the host

cell to direct the polypeptide to the cell surface or secrete the polypeptide into the media, and this signal peptide is clipped off by the host cell before the protein leaves the cell. Signal sequences can be found associated with a variety of proteins native to prokaryotes and eukaryotes.

The term "oligonucleotide," as used herein in referring to the probe of the present invention, is defined as a molecule comprised of two or more ribonucleotides, preferably more than three. Its exact size will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide.

The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product, which is complementary to a nucleic acid strand, is induced, i.e., in the presence of nucleotides and an inducing agent such as a DNA polymerase and at a suitable temperature and pH. The primer may be either single-stranded or double-stranded and must be sufficiently long to prime the synthesis of the desired extension product in the presence of the inducing agent. The exact length of the primer will depend upon many factors, including temperature, source of primer and use of the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides.

The primers herein are selected to be "substantially" complementary to different strands of a particular target DNA sequence. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand.

Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to hybridize therewith and thereby form the template for the synthesis of the extension product.

A "heterologous" region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

A cell has been "transformed" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived

from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

Two DNA sequences are "substantially homologous" when at least about 75% (preferably at least about 80%, and most preferably at least about 90 or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Maniatis et al., *supra*; DNA Cloning, Vols. I & II, *supra*; Nucleic Acid Hybridization, *supra*.

It should be appreciated that also within the scope of the present invention are DNA sequences encoding alpha kinase, selected from the group of melanoma kinase, kidney kinase, heart kinase, skeletal muscle kinase and lymphocyte kinase, which code for a protein having the same amino acid sequence as any of SEQ ID NOS:, but which are degenerate to any of SEQ ID NOS:. By "degenerate to" is meant that a different three-letter codon is used to specify a particular amino acid. It is well known in the art that the following codons can be used interchangeably to code for each specific amino acid:

Phenylalanine (Phe or F)	UUU or UUC
Leucine (Leu or L)	UUA or UUG or CUU or CUC or CUA or CUG
Isoleucine (Ile or I)	AUU or AUC or AUA
Methionine (Met or M)	AUG
Valine (Val or V)	GUU or GUC or GUA or GUG
Serine (Ser or S)	UCU or UCC or UCA or UCG or AGU or AGC
Proline (Pro or P)	CCU or CCC or CCA or CCG
Threonine (Thr or T)	ACU or ACC or ACA or ACG



Alanine (Ala or A)	GCU or GCG or GCA or GCG
Tyrosine (Tyr or Y)	UAU or UAC
Histidine (His or H)	CAU or CAC
Glutamine (Gln or Q)	CAA or CAG
Asparagine (Asn or N)	AAU or AAC
Lysine (Lys or K)	AAA or AAG
Aspartic Acid (Asp or D)	GAU or GAC
Glutamic Acid (Glu or E)	GAA or GAG
Cysteine (Cys or C)	UGU or UGC
Arginine (Arg or R)	CGU or CGC or CGA or CGG or AGA or AGG
Glycine (Gly or G)	GGU or GGC or GGA or GGG
Tryptophan (Trp or W)	UGG
Termination codon	UAA (ochre) or UAG (amber) or UGA (opal)

It should be understood that the codons specified above are for RNA sequences. The corresponding codons for DNA have a T substituted for U.

Mutations can be made in any of SEQ ID NOS: such that a particular codon is changed to a codon which codes for a different amino acid. Such a mutation is generally made by making the fewest nucleotide changes possible. A substitution mutation of this sort can be made to change an amino acid in the resulting protein in a non-conservative manner (*i.e.*, by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to another grouping) or in a conservative manner (*i.e.*, by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to the same grouping). Such a conservative change generally leads to less change in the structure and function of the resulting protein. A non-conservative change is more likely to alter the structure, activity or function of the resulting protein. The present invention should be

considered to include sequences containing conservative changes which do not significantly alter the activity or binding characteristics of the resulting protein.

The following is one example of various groupings of amino acids:

(I) Amino acids with nonpolar R groups: Alanine, Valine, Leucine, Isoleucine, Proline, Phenylalanine, Tryptophan, Methionine; (II) Amino acids with uncharged polar R groups: Glycine, Serine, Threonine, Cysteine, Tyrosine, Asparagine, Glutamine; (III) Amino acids with charged polar R groups (negatively charged at pH 6.0): Aspartic acid, Glutamic acid; (IV) Basic amino acids (positively charged at pH 6.0): Lysine, Arginine, Histidine (at pH 6.0).

Another grouping may be those amino acids with phenyl groups:

Phenylalanine, Tryptophan, Tyrosine

Another grouping may be according to molecular weight (i.e., size of R groups):

Glycine	75
Alanine	89
Serine	105
Proline	115
Valine	117
Threonine	119
Cysteine	121
Leucine	131
Isoleucine	131
Asparagine	132
Aspartic acid	133
Glutamine	146
Lysine	146
Glutamic acid	147
Methionine	149

Histidine (at pH 6.0)	155
Phenylalanine	165
Arginine	174
Tyrosine	181
Tryptophan	204

Particularly preferred substitutions are:

- Lys for Arg and vice versa such that a positive charge may be maintained;
- Glu for Asp and vice versa such that a negative charge may be maintained;
- Ser for Thr such that a free -OH can be maintained; and
- Gln for Asn such that a free NH<sub>2</sub> can be maintained.

Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced a potential site for disulfide bridges with another Cys. A His may be introduced as a particularly "catalytic" site (*i.e.*, His can act as an acid or base and is the most common amino acid in biochemical catalysis). Pro may be introduced because of its particularly planar structure, which induces  $\beta$ -turns in the protein's structure.

Another feature of this invention is the expression of the DNA sequences disclosed herein. As is well known in the art, DNA sequences may be expressed by operatively linking them to an expression control sequence in an appropriate expression vector and employing that expression vector to transform an appropriate unicellular host.

Such operative linking of a DNA sequence of this invention to an expression control sequence, of course, includes, if not already part of the DNA sequence, the provision of an initiation codon, ATG, in the correct reading frame upstream of the DNA sequence.

A wide variety of host/expression vector combinations may be employed in expressing the DNA sequences of this invention. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, e.g., *E. coli* plasmids col El, pCR1, pBR322, pMB9 and their derivatives, plasmids such as RP4; phage DNAs, e.g., the numerous derivatives of phage  $\lambda$ , e.g., NM989, and other phage DNA, e.g., M13 and filamentous single stranded phage DNA; yeast plasmids such as the 2 $\mu$  plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; and the like.

Any of a wide variety of expression control sequences -- sequences that control the expression of a DNA sequence operatively linked to it -- may be used in these vectors to express the DNA sequences of this invention. Such useful expression control sequences include, for example, the early or late promoters of SV40, CMV, vaccinia, polyoma or adenovirus, the *lac* system, the *trp* system, the *TAC* system, the *TRC* system, the *LTR* system, the major operator and promoter regions of phage  $\lambda$ , the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase (e.g., Pho5), the promoters of the yeast  $\alpha$ -mating factors, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

A wide variety of unicellular host cells are also useful in expressing the DNA sequences of this invention. These hosts may include well known eukaryotic and prokaryotic hosts, such as strains of *E. coli*, *Pseudomonas*, *Bacillus*, *Streptomyces*, fungi such as yeasts, and animal cells, such as CHO, R1.1, B-W and L-M cells, African Green Monkey kidney cells (e.g., COS 1, COS 7, BSC1, BSC40, and BMT10), insect cells (e.g., Sf9), and human cells and plant cells in tissue culture.

It will be understood that not all vectors, expression control sequences and hosts will function equally well to express the DNA sequences of this invention. Neither will all hosts function equally well with the same expression system. However, one skilled in the art will be able to select the proper vectors, expression control sequences, and hosts without undue experimentation to accomplish the desired expression without departing from the scope of this invention. For example, in selecting a vector, the host must be considered because the vector must function in it. The vector's copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, will also be considered.

In selecting an expression control sequence, a variety of factors will normally be considered. These include, for example, the relative strength of the system, its controllability, and its compatibility with the particular DNA sequence or gene to be expressed, particularly as regards potential secondary structures. Suitable unicellular hosts will be selected by consideration of, e.g., their compatibility with the chosen vector, their secretion characteristics, their ability to fold proteins correctly, and their fermentation requirements, as well as the toxicity to the host of the product encoded by the DNA sequences to be expressed, and the ease of purification of the expression products.

Considering these and other factors, a person skilled in the art will be able to construct a variety of vector/expression control sequence/host combinations that will express the DNA sequences of this invention on fermentation or in large scale animal culture.

The present invention extends to the preparation of antisense oligonucleotides and ribozymes that may be used to interfere with the expression of the eEF-2 kinase gene at the translational level. This approach utilizes antisense nucleic acid and ribozymes to block translation of a specific mRNA, either by masking that mRNA with an antisense nucleic acid or cleaving it with a ribozyme.

Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule. (See Weintraub, 1990; Marcus-Sekura, 1988.) In the cell, they hybridize to that mRNA, forming a double stranded molecule. The cell does not translate an mRNA in this double-stranded form. Therefore, antisense nucleic acids interfere with the expression of mRNA into protein. Oligomers of about fifteen nucleotides and molecules that hybridize to the AUG initiation codon will be particularly efficient, since they are easy to synthesize and are likely to pose fewer problems than larger molecules when introducing them into eEF-2 kinase-producing cells. Antisense methods have been used to inhibit the expression of many genes *in vitro* (Marcus-Sekura, 1988; Hambor et al., 1988).

Ribozymes are RNA molecules possessing the ability to specifically cleave other single stranded RNA molecules in a manner somewhat analogous to DNA restriction endonucleases. Ribozymes were discovered from the observation that certain mRNAs have the ability to excise their own introns. By modifying the nucleotide sequence of these RNAs, researchers have been able to engineer molecules that recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech, 1988.). Because they are sequence-specific, only mRNAs with particular sequences are inactivated.

Investigators have identified two types of ribozymes, *Tetrahymena*-type and "hammerhead"-type. (Hasselhoff and Gerlach, 1988) *Tetrahymena*-type ribozymes recognize four-base sequences, while "hammerhead"-type recognize eleven- to eighteen-base sequences. The longer the recognition sequence, the more likely it is to occur exclusively in the target mRNA species. Therefore, hammerhead-type ribozymes are preferable to *Tetrahymena*-type ribozymes for inactivating a specific mRNA species, and eighteen base recognition sequences are preferable to shorter recognition sequences.

### Polypeptides

In a further aspect, the present invention includes an isolated protein characterized by the presence of at least two domains, one of the domains being an alpha-kinase catalytic domain and the other domain being an ion channel domain.

Thus, the present invention provides an isolated melanoma alpha kinase protein characterized by having an alpha-kinase catalytic domain and an ion channel domain. In particular, a melanoma alpha kinase protein is provided which comprises the amino acid sequence set out in SEQ ID NO: 27 and 29, and analogs, variants and fragments thereof.

The invention further provides an isolated kidney alpha kinase protein characterized by having an alpha-kinase catalytic domain and an ion channel domain. In particular, the kidney alpha kinase protein comprises the amino acid sequence set out in SEQ ID NO: 31 and 33, and analogs, variants and fragments thereof.

The present invention further provides an isolated heart alpha kinase protein. In particular, the heart alpha kinase protein comprises the amino acid sequence set out in SEQ ID NO: 35 and 37, and analogs, variants and immunogenic fragments thereof.

The present invention still further provides an isolated skeletal muscle alpha kinase protein. In particular, the skeletal muscle alpha kinase protein comprises the amino acid sequence set out in SEQ ID NO: 39, and analogs, variants and immunogenic fragments thereof.

The invention includes an isolated lymphocyte alpha kinase protein. In particular, the lymphocyte alpha kinase protein comprises the amino acid sequence set out in SEQ ID NO: 41, and analogs, variants and immunogenic fragments thereof.

Two amino acid sequences are "substantially homologous" when at least about 70% of the amino acid residues (preferably at least about 80%, and most preferably at least about 90 or 95%) are identical, or represent conservative substitutions.

Antibodies

In a further aspect, the invention provides a purified antibody to an alpha kinase protein selected from the group of melanoma alpha kinase, kidney alpha kinase, heart alpha kinase, skeletal muscle alpha kinase and lymphocyte alpha kinase.

A monoclonal antibody to an alpha kinase protein selected from the group of melanoma alpha kinase, kidney alpha kinase, heart alpha kinase, skeletal muscle alpha kinase and lymphocyte alpha kinase is still further provided. the invention includes an immortal cell line that produces a monoclonal antibody to an alpha kinase protein selected from the group of melanoma alpha kinase, kidney alpha kinase, heart alpha kinase, skeletal muscle alpha kinase and lymphocyte alpha kinase.

Any such contemplated antibody may be labeled with a detectable label. The label may be selected from the group consisting of an enzyme, a chemical which fluoresces, and a radioactive element.

The invention further includes an antibody to an alpha kinase protein selected from the group of melanoma alpha kinase, kidney alpha kinase, heart alpha kinase, skeletal muscle alpha kinase and lymphocyte alpha kinase, which recognizes the phosphorylated form of the alpha kinase or a phosphorylated fragment thereof.

The present invention likewise extends to antibodies against specifically phosphorylated alpha kinase targets, including naturally raised and recombinantly prepared antibodies. These antibodies and their labeled counterparts are included within the scope of the present invention for their particular ability in detecting alpha kinase activity *via* detection of the phosphorylated product by ELISA or any other immunoassay known to the skilled artisan.

In the instance where a radioactive label, such as the isotopes  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{33}\text{P}$ ,  $^{35}\text{S}$ ,  $^{36}\text{Cl}$ ,  $^{51}\text{Cr}$ ,  $^{57}\text{Co}$ ,  $^{58}\text{Co}$ ,  $^{59}\text{Fe}$ ,  $^{90}\text{Y}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ , and  $^{186}\text{Re}$  are used, known currently available



counting procedures may be utilized. In the instance where the label is an enzyme, detection may be accomplished by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques known in the art.

An "antibody" is any immunoglobulin, including antibodies and fragments thereof, that binds a specific epitope. The term encompasses polyclonal, monoclonal, and chimeric antibodies, the last mentioned described in further detail in U.S. Patent Nos. 4,816,397 and 4,816,567.

An "antibody combining site" is that structural portion of an antibody molecule comprised of heavy and light chain variable and hypervariable regions that specifically binds antigen.

The phrase "antibody molecule" in its various grammatical forms as used herein contemplates both an intact immunoglobulin molecule and an immunologically active portion of an immunoglobulin molecule.

Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contains the paratope, including those portions known in the art as Fab, Fab',  $F(ab')_2$  and  $F(v)$ , which portions are preferred for use in the therapeutic methods described herein.

Fab and  $F(ab')_2$  portions of antibody molecules are prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibody molecules by methods that are well-known. See for example, U.S. Patent No. 4,342,566 to Theofilopolous et al. Fab' antibody molecule portions are also well-known and are produced from  $F(ab')_2$  portions followed by reduction of the disulfide bonds linking the two heavy chain portions as with mercaptoethanol, and followed by alkylation of

the resulting protein mercaptan with a reagent such as iodoacetamide. An antibody containing intact antibody molecules is preferred herein.

The phrase "monoclonal antibody" in its various grammatical forms refers to an antibody having only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal antibody thus typically displays a single binding affinity for any antigen with which it immunoreacts. A monoclonal antibody may therefore contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different antigen; e.g., a bispecific (chimeric) monoclonal antibody.

In a particular embodiment, the present invention relates to phosphorylation target analogs, which are short peptide sequences derived from phosphorylation targets of this new superfamily of protein alpha kinases centered around the alpha kinases selected from the group of melanoma kinase, kidney kinase, heart kinase, skeletal muscle kinase and lymphocyte kinase. Specifically, it is contemplated that these peptide analogs will be instrumental in the development of high throughput screening assays to identify inhibitors of members of this new superfamily.

Also, antibodies including both polyclonal and monoclonal antibodies, and drugs that modulate the production or activity of alpha kinase may possess certain diagnostic applications and may, for example, be utilized for the purpose of detecting and/or measuring levels of alpha kinase. It is anticipated that further experimentation will reveal a prognostic correlation between alpha kinase levels and the prediction and or progression of certain malignancies associated with carcinoma. For example, alpha kinase may be used to produce both polyclonal and monoclonal antibodies to themselves in a variety of cellular media, by known techniques such as the hybridoma technique utilizing, for example, fused mouse spleen lymphocytes and myeloma cells.

Likewise, small molecules that mimic or antagonize the activity of alpha kinase of the invention may be discovered or synthesized, and may be used in diagnostic and/or therapeutic protocols.

The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal, antibody-producing cell lines can also be created by techniques other than fusion, such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, e.g., M. Schreier et al., "Hybridoma Techniques" (1980); Hammerling et al., "Monoclonal Antibodies And T-cell Hybridomas" (1981); Kennett et al., "Monoclonal Antibodies" (1980); see also U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,451,570; 4,466,917; 4,472,500; 4,491,632; 4,493,890.

Panels of monoclonal antibodies produced against alpha kinase peptides can be screened for various properties; *i.e.*, isotype, epitope, affinity, etc. Of particular interest are monoclonal antibodies that neutralize the activity of alpha kinase. Such monoclonals can be readily identified in alpha kinase activity assays. High affinity antibodies are also useful when immunoaffinity purification of native or recombinant alpha kinase is desired.

Preferably, the anti-alpha kinase antibody used in the diagnostic methods of this invention is an affinity purified polyclonal antibody. More preferably, the antibody is a monoclonal antibody (mAb). In addition, it is preferable for the anti-alpha kinase antibody molecules used herein be in the form of Fab, Fab', F(ab')<sub>2</sub> or F(v) portions of whole antibody molecules.

As suggested earlier, the diagnostic method of the present invention comprises examining a cellular sample or medium by means of an assay including an effective amount of an antagonist to alpha kinase, such as an anti-alpha kinase antibody, preferably an affinity-purified polyclonal antibody, and more preferably a mAb. In

addition, it is preferable for the anti-alpha kinase antibody molecules used herein be in the form of Fab, Fab', F(ab')<sub>2</sub> or F(v) portions or whole antibody molecules. As previously discussed, patients capable of benefitting from this method include those suffering from cancer, a pre-cancerous lesion, a viral infection or other like pathological derangement. Methods for isolating the alpha kinase and inducing anti-alpha kinase antibodies and for determining and optimizing the ability of anti-alpha kinase antibodies to assist in the examination of the target cells are all well-known in the art.

Methods for producing polyclonal anti-polypeptide antibodies are well-known in the art. See U.S. Patent No. 4,493,795 to Nestor et al. A monoclonal antibody, typically containing Fab and/or F(ab')<sub>2</sub> portions of useful antibody molecules, can be prepared using the hybridoma technology described in *Antibodies - A Laboratory Manual*, Harlow and Lane, eds., Cold Spring Harbor Laboratory, New York (1988), which is incorporated herein by reference.

Splenocytes are typically fused with myeloma cells using polyethylene glycol (PEG) 6000. Fused hybrids are selected by their sensitivity to HAT. Hybridomas producing a monoclonal antibody useful in practicing this invention are identified by their ability to immunoreact with a particular kinase and of the present invention and their ability to inhibit specified alpha kinase activity in target cells.

A monoclonal antibody useful in practicing the present invention can be produced by initiating a monoclonal hybridoma culture comprising a nutrient medium containing a hybridoma that secretes antibody molecules of the appropriate antigen specificity. The culture is maintained under conditions and for a time period sufficient for the hybridoma to secrete the antibody molecules into the medium. The antibody-containing medium is then collected. The antibody molecules can then be further isolated by well-known techniques.

Media useful for the preparation of these compositions are both well-known in the art and commercially available and include synthetic culture media, inbred mice and the like. An exemplary synthetic medium is Dulbecco's minimal essential medium (DMEM; Dulbecco et al., *Virology* **8**:396 (1959)) supplemented with 4.5 gm/l glucose, 20mM glutamine, and 20% fetal calf serum. An exemplary inbred mouse strain is the Balb/c.

Methods for producing monoclonal anti-alpha kinase antibodies are also well-known in the art. See Niman et al., *Proc. Natl. Acad. Sci. USA*, **80**:4949-4953 (1983).

Typically, the present alpha kinase or a peptide analog is used either alone or conjugated to an immunogenic carrier, as the immunogen in the before described procedure for producing anti-alpha kinase monoclonal antibodies. The hybridomas are screened for the ability to produce an antibody that immunoreacts with the eEF-2 kinase peptide analog and the present alpha kinase.

#### Therapeutic Compositions and Methods

Therapeutic possibilities are raised by the knowledge of the alpha kinase sequences, melanoma kinase, kidney kinase, heart kinase, skeletal muscle kinase and lymphocyte kinase. Accordingly, it is contemplated that sequences that are derived from the complement to the alpha kinase mRNA sequence, and various modifications thereof, can act as potent antisense drugs that either inhibit expression in a competitive fashion, or, more effectively, by nuclease activity associated with the antisense drug that cleaves the alpha kinase mRNA sequence, thus rendering it irreversibly inactive. Alternative therapeutics are also contemplated that concern the use of peptides and peptide analogs representing portions of phosphorylation target amino acid sequences. It is envisioned that such peptide-based drugs would inhibit alpha kinase activity on its native target, thus bypassing the cascade of events that would lead to malignant transformation.

In a particular aspect, the present invention includes a pharmaceutical composition comprising one or more alpha kinase protein selected from the group of melanoma alpha kinase, kidney alpha kinase, heart alpha kinase, skeletal muscle alpha kinase and lymphocyte alpha kinase, and a pharmaceutically acceptable carrier.

The present invention provides a method for treating an animal in need of increased activity of melanoma alpha kinase which comprises administration of melanoma alpha kinase to the animal.

The present invention further provides a method for treating an animal in need of increased activity of melanoma alpha kinase which comprises administration of an antibody against melanoma alpha kinase to the animal.

The present invention also provides a method for treating an animal in need of increased activity of kidney alpha kinase which comprises administration of kidney alpha kinase to the animal.

The invention also includes a method for treating an animal in need of increased activity of kidney alpha kinase which comprises administration of an antibody against kidney alpha kinase to the animal.

The invention further provides a method for treating an animal in need of increased activity of heart alpha kinase which comprises administration of heart alpha kinase to the animal.

The present invention also contemplates a method for treating an animal in need of increased activity of heart alpha kinase which comprises administration of an antibody against heart alpha kinase to the animal.

In an additional aspect, the invention provides a method for treating an animal in need of increased activity of skeletal muscle alpha kinase which comprises administration of skeletal muscle alpha kinase to the animal.

A method for treating an animal in need of increased activity of skeletal muscle alpha kinase which comprises administration of an antibody against skeletal muscle alpha kinase to the animal is further provided.

The present invention includes method for treating an animal in need of increased activity of lymphocyte alpha kinase which comprises administration of lymphocyte alpha kinase to the animal.

The present invention further provides a method for treating an animal in need of increased activity of lymphocyte alpha kinase which comprises administration of an antibody against lymphocyte alpha kinase to the animal.

The therapeutic method provided herein could include the method for the treatment of various pathologies or other cellular dysfunctions and derangements by the administration of pharmaceutical compositions that may comprise effective inhibitors of alpha kinase activity, or other equally effective drugs developed for instance by a drug screening assay prepared and used in accordance with a further aspect of the present invention.

It is a still further object of the present invention to provide a method for the treatment of mammals to control the amount or activity of alpha kinase, so as to alter the adverse consequences of such presence or activity, or where beneficial, to enhance such activity.

It is a still further object of the present invention to provide a method for the treatment of mammals to control the amount or activity of alpha kinase, so as to treat or avert the adverse consequences of invasive, spontaneous or idiopathic pathological states.

The present invention further contemplates therapeutic compositions useful in practicing the therapeutic methods of this invention. A subject therapeutic composition includes, in admixture, a pharmaceutically acceptable excipient (carrier) and one or more of an anti-alpha kinase antibody, peptide analog capable of competing for phosphorylation of target by alpha kinase, antisense drug against alpha kinase mRNA, or any other compound that is found to inhibit alpha kinase activity. In a preferred embodiment, the composition comprises an antigen capable of modulating the activity of alpha kinase within a target cell.

The preparation of therapeutic compositions which contain polypeptides, analogs or active fragments as active ingredients is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions, however, solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified. The active therapeutic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents which enhance the effectiveness of the active ingredient.

A polypeptide, analog or active fragment can be formulated into the therapeutic composition as neutralized pharmaceutically acceptable salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide or antibody molecule) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as



isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The therapeutic polypeptide-, analog- or active fragment-containing compositions are conventionally administered intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for humans, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; *i.e.*, carrier, or vehicle.

The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, capacity of the subject's immune system to utilize the active ingredient, and degree of inhibition or neutralization of eEF-2 kinase activity desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosages may range from about 0.1 to 20, preferably about 0.5 to about 10, and more preferably one to several, milligrams of active ingredient per kilogram body weight of individual per day and depend on the route of administration. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations of ten nanomolar to ten micromolar in the blood are contemplated.

### Formulations

#### Intravenous Formulation I

Ingredient

mg/ml

cefotaxime	250.0
antibody, peptide, antisense drug, or other compound	10.0
dextrose USP	45.0
sodium bisulfite USP	3.2
edetate disodium USP	0.1
water for injection q.s.a.d.	1.0 ml

#### Intravenous Formulation II

<u>Ingredient</u>	<u>mg/ml</u>
ampicillin	250.0
antibody, peptide, antisense drug, or other compound	10.0
sodium bisulfite USP	3.2
disodium edetate USP	0.1
water for injection q.s.a.d.	1.0 ml

#### Intravenous Formulation III

<u>Ingredient</u>	<u>mg/ml</u>
gentamicin (charged as sulfate)	40.0
antibody, peptide, antisense drug, or other compound	10.0
sodium bisulfite USP	3.2
disodium edetate USP	0.1
water for injection q.s.a.d.	1.0 ml

#### Intravenous Formulation IV

<u>Ingredient</u>	<u>mg/ml</u>
antibody, peptide, antisense drug, or other compound	10.0
dextrose USP	45.0
sodium bisulfite USP	3.2
edetate disodium USP	0.1
water for injection q.s.a.d.	1.0 ml

As used herein, "pg" means picogram, "ng" means nanogram, "ug" or "μg" mean microgram, "mg" means milligram, "ul" or "μl" mean microliter, "ml" means milliliter, "l" means liter.

The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human.

The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to prevent, and preferably reduce by at least about 30 percent, more preferably by at least 50 percent, most preferably by at least 90 percent, a clinically significant change in the S phase activity of a target cellular mass, or other feature of pathology such as for example, elevated blood pressure, fever or white cell count as may attend its presence and activity.

#### Assays and Methods

The present invention also relates to a variety of diagnostic applications, including methods for detecting and quantifying the levels of alpha kinase. As mentioned earlier, alpha kinase can be used to produce antibodies to itself by a variety of known techniques, and such antibodies could then be isolated and utilized as in tests for the presence and levels of alpha kinase activity in suspect target cells.

The invention includes an assay system for screening of potential drugs effective at attenuating alpha kinase activity of target mammalian cells by interrupting or potentiating the phosphorylation of alpha kinase selected from the group of melanoma kinase, heart kinase, kidney kinase, skeletal muscle kinase and lymphocyte kinase. In

one instance, the test drug could be administered to a cellular sample along with ATP carrying a detectable label on its  $\gamma$ -phosphate that gets transferred to the kinase target, including the kinase itself, or a peptide substrate, by the particular alpha kinase. Quantification of the labeled kinase target or peptide substrate is diagnostic of the candidate drug's efficacy. A further embodiment would provide for the assay to be performed using a purely *in vitro* system comprised of the alpha kinase, ATP or labeled ATP, the kinase target or peptide substrate, appropriate buffer, and detection reagents and/or instrumentation to detect and quantify the extent of alpha kinase-directed phosphorylation activity.

The assay system could more importantly be adapted to identify drugs or other entities that are capable of binding to the alpha kinase and/or its cognate phosphorylation target, either in the cytoplasm or in the nucleus, thereby inhibiting or potentiating alpha kinase activity and its resultant phenotypic outcome. Such an assay would be useful in the development of drugs that would be specific against particular cellular activity, or that would potentiate such activity, in time or in level of activity. For example, such drugs might be used to treat various carcinomas or other hyperproliferative pathologies.

In an additional aspect, the present invention includes a method for detecting the presence or activity of an alpha kinase protein selected from the group of melanoma alpha kinase, kidney alpha kinase, heart alpha kinase, skeletal muscle alpha kinase and lymphocyte alpha kinase, wherein said alpha kinase is measured by:

A. contacting a biological sample from a mammal in which the presence or activity of said alpha kinase is suspected with a binding partner of said alpha kinase under conditions that allow binding of said alpha kinase to said binding partner to occur; and

B. detecting whether binding has occurred between said alpha kinase from said sample and the binding partner;

wherein the detection of binding indicates that presence or activity of said alpha kinase in said sample.

The present invention further provides a method for detecting the presence of an alpha kinase protein selected from the group of melanoma alpha kinase, kidney alpha kinase, heart alpha kinase, skeletal muscle alpha kinase and lymphocyte alpha kinase, wherein the alpha kinase is measured by:

- a. contacting a sample in which the presence or activity of an alpha kinase protein selected from the group of melanoma alpha kinase, kidney alpha kinase, heart alpha kinase, skeletal muscle alpha kinase and lymphocyte alpha kinase is suspected with an antibody to the said alpha kinase protein under conditions that allow binding of the alpha kinase protein to the binding partner to occur; and
- b. detecting whether binding has occurred between the alpha kinase protein from the sample and the antibody;

wherein the detection of binding indicates the presence or activity of the alpha kinase protein in the sample.

In a still further aspect, the invention provides a method of testing the ability of a drug or other entity to modulate the kinase activity of an alpha kinase protein selected from the group of melanoma alpha kinase, kidney alpha kinase, heart alpha kinase, skeletal muscle alpha kinase and lymphocyte alpha kinase which comprises:

- A. culturing a colony of test cells containing the alpha kinase protein;
- B. adding the drug or other entity under test; and
- C. measuring the kinase activity of said alpha kinase protein in the test

cells, wherein when the amount of kinase activity in the presence of the modulator is greater than in its absence, the modulator is identified as an agonist or activator of the alpha kinase protein, whereas when the amount of kinase activity in the presence of the modulator is less than in its absence, the modulator is identified as an antagonist or inhibitor of the alpha kinase protein.

It is a further object of the present invention to provide a method for detecting alpha kinase activity in mammals in which invasive, spontaneous, or idiopathic pathological states are suspected to be present.

As described in detail above, antibody(ies) to alpha kinase can be produced and isolated by standard methods including the well known hybridoma techniques. For convenience, the antibody(ies) to alpha kinase will be referred to herein as Ab<sub>1</sub> and antibody(ies) raised in another species as Ab<sub>2</sub>.

The presence and levels of alpha kinase in cells can be ascertained by the usual immunological procedures applicable to such determinations. A number of useful procedures are known. Three such procedures which are especially useful, utilize either alpha kinase labeled with a detectable label, antibody Ab<sub>1</sub> labeled with a detectable label, or antibody Ab<sub>2</sub> labeled with a detectable label. The procedures may be summarized by the following equations wherein the asterisk indicates that the particle is labeled, and "~" stands for alpha kinase:

$$A. \sim^* + Ab_1 = \sim^* Ab_1$$

$$B. \sim + Ab^* = \sim Ab^*$$

$$C. \sim + Ab_1 + Ab_2^* = \sim Ab_1 Ab_2^*$$

The procedures and their application are all familiar to those skilled in the art and accordingly may be utilized within the scope of the present invention. The "competitive" procedure, Procedure A, is described in U.S. Patent Nos. 3,654,090 and 3,850,752. Procedure C, the "sandwich" procedure, is described in U.S. Patent Nos. RE 31,006 and 4,016,043. Still other procedures are known such as the "double antibody," or "DASP" procedure.

In each instance, alpha kinase forms complexes with one or more antibody(ies) or binding partners and one member of the complex is labeled with a detectable label. The fact that a complex has formed and, if desired, the amount thereof, can be determined by known methods applicable to the detection of labels.

It will be seen from the above, that a characteristic property of  $Ab_2$  is that it will react with  $Ab_1$ . This is because  $Ab_1$  raised in one mammalian species has been used in another species as an antigen to raise the antibody  $Ab_2$ . For example,  $Ab_2$  may be raised in goats using rabbit antibodies as antigens.  $Ab_2$  therefore would be anti-rabbit antibody raised in goats. For purposes of this description and claims,  $Ab_1$  will be referred to as a primary or anti-alpha kinase antibody, and  $Ab_2$  will be referred to as a secondary or anti- $Ab_1$  antibody.

The labels most commonly employed for these studies are radioactive elements, enzymes, chemicals which fluoresce when exposed to ultraviolet light, and others.

A number of fluorescent materials are known and can be utilized as labels. These include, for example, fluorescein, rhodamine, auramine, Texas Red, AMCA blue and Lucifer Yellow. A particular detecting material is anti-rabbit antibody prepared in goats and conjugated with fluorescein through an isothiocyanate.

eEF-2 kinase can also be labeled with a radioactive element or with an enzyme. The radioactive label can be detected by any of the currently available counting procedures. The preferred isotope may be selected from  $^3H$ ,  $^{14}C$ ,  $^{32}P$ ,  $^{33}P$ ,  $^{35}S$ ,  $^{36}Cl$ ,  $^{51}Cr$ ,  $^{57}Co$ ,  $^{58}Co$ ,  $^{59}Fe$ ,  $^{90}Y$ ,  $^{125}I$ ,  $^{131}I$ , and  $^{186}Re$ .

Enzyme labels are likewise useful, and can be detected by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric, amperometric or gasometric techniques. The enzyme is conjugated to the selected particle by reaction with bridging molecules such as carbodiimides, diisocyanates, glutaraldehyde and the like. Many enzymes which can be used in these procedures are known and can be utilized. The preferred are peroxidase,  $\beta$ -glucuronidase,  $\beta$ -D-glucosidase,  $\beta$ -D-galactosidase, urease, glucose oxidase plus peroxidase and alkaline phosphatase. U.S. Patent Nos. 3,654,090; 3,850,752; and 4,016,043 are referred to by way of example for their disclosure of alternate labeling material and methods.

A particular assay system developed and utilized in accordance with the present invention, is known as a receptor assay. In a receptor assay, the material to be assayed is appropriately labeled and then certain cellular test colonies are inoculated with a quantity of both the labeled and unlabeled material after which binding studies are conducted to determine the extent to which the labeled material binds to the cell receptors. In this way, differences in affinity between materials can be ascertained.

Accordingly, a purified quantity of the alpha kinase may be radiolabeled and combined, for example, with antibodies or other inhibitors thereto, after which binding studies would be carried out. Solutions would then be prepared that contain various quantities of labeled and unlabeled uncombined alpha kinase, and cell samples would then be inoculated and thereafter incubated. The resulting cell monolayers are then washed, solubilized and then counted in a gamma counter for a length of time sufficient to yield a standard error of <5%. These data are then subjected to Scatchard analysis after which observations and conclusions regarding material activity can be drawn. While the foregoing is exemplary, it illustrates the manner in which a receptor assay may be performed and utilized, in the instance where the cellular binding ability of the assayed material may serve as a distinguishing characteristic.

In accordance with the above, an assay system for screening potential drugs effective to modulate the activity of alpha kinase may be prepared. The alpha kinase may be introduced into a test system, and the prospective drug may also be introduced into the resulting cell culture, and the culture thereafter examined to observe any changes in the alpha kinase activity of the cells, due either to the addition of the prospective drug alone, or due to the effect of added quantities of the known alpha kinase. Alternatively, these assays can be carried out in a purely *in vitro* fashion as discussed below.

The invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention. The following examples



are presented in order to more fully illustrate the preferred embodiments of the invention and should in no way be construed, however, as limiting the broad scope of the invention.

### EXAMPLE 1

#### MOLECULAR CLONING OF cDNAs ENCODING *C. elegans*, MOUSE, RAT, AND HUMAN eEF-2 KINASES

eEF-2 kinase from rabbit reticulocyte lysate was purified as described (Hait et al., (1996) *FEBS Lett.* 397:55-60). Peptides were generated from the nitrocellulose-bound 103-kDa eEF-2 kinase protein by *in situ* tryptic digestion (Erdjument-Bromage et al., (1994) *Protein Sci.* 3:2435-2446) and fractionated by reverse-phase HPLC (Elicone et al., (1994) *J. Chromatogr.* 676:121-137) using a 1.0 mm Reliasil C18 column. Selected peak fraction were then analyzed by a combination of automated Edman sequencing and matrix-assisted laser-desorption time-of-flight mass spectrometry (Erdjument-Bromage et al., (1994)). The peptide sequences provided an essential lead into the cloning of eEF-2 kinase from human, mouse, rat, and *Caenorhabditis elegans*.

To clone the cDNA for *C. elegans* eEF-2 kinase, oligonucleotide primers were designed based on the amino and carboxy termini of the predicted gene product from F42A10.4. Reverse transcriptase-PCR (RT-PCR) was performed using these primers and total RNA from *C. elegans*. A single PCR product of ~2.3 kb was obtained and gel-purified using a gel extraction kit (Qiagen, Chatsworth, CA). The fragment was ligated into vector pCR2.1 using the TA cloning kit (Invitrogen, Sorrento Valley, CA), and then transformed into *Escherichia coli*. Plasmid DNA was purified, and restriction analysis used to verify the orientation of the coding sequence with respect to the T7 promoter. Two clones (*Cefk-1* and *Cefk-2*, *C. elegans* eEF-2 kinase isoforms 1 and 2) were chosen and sequenced using a Li-Cor (Lincoln, NE) Long Read IR model 400L Automated DNA Sequencer. Analysis revealed that the two

clones were identical except for a deletion of 24 bp in *Cefk-2* which corresponds to exon 4 and probably represents an alternatively spliced form.

To clone the mouse eEF-2 kinase, degenerate primers were designed based on the amino acid sequence of two peptides from rabbit eEF-2 kinase (LTPQAFSHFTFER (SEQ ID NO: 15) and LANXYYEKAE (SEQ ID NO: 16)); primer A, CA(G/A)GC(C/G/T/A)TT(C/T)(T/A)(C/G)(T/CCA(C/T)TT(C/T)AC(C/G/T/A)TT(C/T)GA(G/A(C/A)G (SEQ ID NO: 17); and primer B, TC(C/G/T/A)GC(C/T)TT(C/T)TC(G/A)TA(G/A)TA(C/T)TT(G/A)TT(C/G/A/T)GC (SEQ ID NO: 18). RT-PCR was performed using primers A and B and poly(A)<sup>+</sup> RNA from mouse spleen (CLONTECH). A single PCR product (~1.6 kb) was cloned into pCR2.1 (Invitrogen) and sequenced. Using sequence information from these mouse eEF-2 kinase cDNA fragments, new primers were designed for 5' rapid amplification of cDNA ends (RACE) and 3' RACE to obtain full-length mouse eEF-2 kinase cDNA. 5' RACE and 3' RACE were performed using Marathon-Ready mouse spleen cDNA (CLONTECH). This was carried out according to the manufacturer's instructions using the primers AP1 and C (TACAATCAGCTGATGACCAGAACGCTC) (SEQ ID NO: 19) 5' antisense, or D (GGATTTGGACTGGACAAGAACCCCC) (SEQ ID NO: 20) 3' sense.

To clone rat eEF-2 kinases, PCR was performed on a rat PC12 cDNA library cloned in λGT10 (CLONTECH) using primer B and vector primers. A 700-bp fragment was specifically amplified. The fragment was cloned into pCR2.1 (Invitrogen) and sequenced. This 700-bp fragment was radiolabeled and used to probe the same PC12 cDNA library (600,000 plaques). Fourteen positives were obtained in the initial screening. Five plaques were chosen for further analysis and sequencing based on insert sizes that ranged from 1.4 to 2.0 kb.

Recently, eEF-2 kinase from rabbit reticulocyte lysate was purified to near homogeneity (Hait et al., (1996)). This enabled determination of its partial amino acid sequence as noted above. Two peptide sequences (LTPQAFSHFTFER (SEQ ID NO: 15) and LANXYYEKAE (SEQ ID NO: 16)) were compared with entries in a nonredundant database using the National Center for Biotechnology Information BLAST program (Altschul et al., (1990) *J. Mol. Biol.* 215:403-410). Matches were found with a *C. elegans* hypothetical protein (F42A10.4; GenBank accession number U10414). This sequence was obtained from the *C. elegans* genome sequencing project and is located on chromosome III (Wilson et al., (1994) *Nature* 368:32-38). The 100% identity between the sequenced peptides and the *C. elegans* protein, as well as the fact that the predicted molecular weight of the *C. elegans* protein is similar to that of eEF-2 kinase, suggested that this gene encoded eEF-2 kinase. We cloned the full-length cDNA by RT-PCR using *C. elegans* total RNA. Several clones were isolated and sequenced. *Cefk-1* has six of the predicted exons and encodes 768 amino acids. *Cefk-2* represents an alternatively spliced form that has five exons; it is missing amino acids 625-632 that correspond to exon four. *Cefk-1* and *Cefk-2* were found to have eEF-2 kinase activity when expressed in cell-free system using a wheat germ extract coupled transcription/translation system.

To determine the amino acid sequence of mammalian eEF-2 kinase, we cloned and sequenced the cDNA of mouse eEF-2 kinase. We reasoned that since the sequenced peptides from rabbit eEF-2 were 100% identical to *C. elegans* eEF-2 kinase, then the two peptides should also match the sequence of mouse eEF-2 kinase. Degenerate primers were designed based on the amino acid sequence of the peptides and were used to perform RT-PCR on mouse spleen poly(A)<sup>+</sup> mRNA. A single PCR product of ~1.6 kb was obtained and sequenced. To obtain the full-length cDNA, 5' RACE and 3' RACE were performed using mouse spleen cDNA. The full-length cDNA, which encodes 724 amino acids, was expressed in a cell-free coupled transcription/translation system. A single translation product with an apparent molecular weight of 100 kDa was obtained.

A cDNA for rat eEF-2 kinase was cloned and sequenced using a fragment of mouse eEF-2 kinase cDNA to probe a PC12 cDNA library. However, after this work was completed, a paper describing the cloning of eEF-2 from rat skeletal muscle was published (Redpath et al., (1996) *J. Biol. Chem.* 271:17547-17554) and the reported sequence appears to be identical to the eEF-2 kinase sequence from PC12 cells. Like the mouse eEF-2 kinase, the rat eEF-2 kinase cDNA encodes a 724-amino acid protein.

The human eEF-2 kinase cDNA was cloned. RT-PCR was performed on poly(A)<sup>+</sup> mRNA from the human glioma cell line T98G using 20'mer primers corresponding to the 5' and 3' ends of the mouse eEF-2 kinase coding region. The human eEF-2 kinase cDNA encodes a 725 amino acid protein.

## EXAMPLE 2

### LACK OF HOMOLOGY OF eEF-2 KINASE TO MEMBERS OF EUKARYOTIC PROTEIN KINASE SUPERFAMILY

The alignment of the amino acid sequences of *C. elegans* and mammalian eEF-2 kinases is shown in FIGURE 2. Rat and mouse eEF-2 kinase are very similar being 97% identical and differing by only 23 amino acids. Human eEF-2 kinase is 90% identical to mouse and rat eEF-2 kinase. In contrast, *C. elegans* eEF-2 kinase is found to be only 40% identical to mammalian eEF-2 kinase.

According to the current classification, eEF-2 kinase belongs to the family of closely related calmodulin-dependent protein kinases. Surprisingly, upon analyzing eEF-2 kinase sequences, we did not find any homology to the other calmodulin-dependent kinases or to any other members of the protein kinase super-family. The only motif which it shares with all other protein kinases is the GXGXXG (SEQ ID NO: 21) motif (279-284 in *C. elegans* eEF-2 kinases; 295-300 in mouse eEF-2 kinase) which forms a glycine-rich loop and is part of the ATP-binding site. Comparison of mammalian and

*C. elegans* eEF-2 kinase revealed only one extended region of homology that spans ~200 amino acids upstream of the GXGXXG motif. The high degree of similarity and the proximity to the nucleotide-binding site suggests that these 200 amino acids represent the catalytic domain. This region has a high degree of similarity and a portion of this region (amino acids 251-300 in mouse eEF-2 kinase) displays 75% identity to the catalytic domain of MHCKA (see below), which also suggests that this is the catalytic domain. In the recently published rat eEF-2 kinase sequence [Redpath *et al.*, *J. Biol. Chem.* **271**: 17547-17554 (1996)], the catalytic domain was predicted to reside between amino acids 288 and 554 based on the homology with the catalytic domain of cAMP-dependant protein kinase (PKA). Our results demonstrate that their prediction cannot be correct for several reasons. First, we find that the homology of this region with PKA is not statistically significant. Second, this region is the least conserved between mammalian and *C. elegans* eEF-2 kinase. Finally, according to secondary structure predictions [made by Alexei V. Finkelstein, Institute of Protein Research, Russia using the ALB-GLOBULE program [Ptitsyn and Finkelstein, *Biopolymers* **22**:15-25 (1983)]], this region most likely has a distorted structure and contains almost no  $\alpha$ -helices or  $\beta$ -strands, which are characteristic of a catalytic domain.

Because eEF-2 kinase is  $Ca^{2+}$ /calmodulin-dependant, it should contain a calmodulin-binding domain, which is usually represented by an amphipathic  $\alpha$ -helix. There are several regions that could possibly assume an amphipathic  $\alpha$ -helical conformation. Further biochemical analysis is required to determine which of these is the calmodulin-binding domain.

In the C-terminal region, there is a short stretch of 22 amino acids which is 86% identical between mammalian and *C. elegans* eEF-2 kinase and is preceded by a longer region of weak homology. We do not know the function of this conserved region at present. One of the possibilities is that it is involved in oligomerization of the kinase. It was thought previously that eEF-2 kinase was an

elongated monomer because it migrated during gel filtration as an ~150-kDa protein and migrated on SDS gels as a 105-kDa polypeptide [Ryazanov and Spirin, *Translational Regulation of Gene Expression*, Plenum, NY, Vol 2, pp 433-455 (1993); Abdelnajid *et al.*, *Int. J. Dev. Biol.*, **37**:279-290 (1993)]. However, the molecular weight of a monomer of mammalian eEF-2 kinase based on the predicted sequence is just 82 kDa. Thus, it is possible that eEF-2 kinase is not a monomer but a responsible for dimerization. Interestingly, according to computer prediction using the COIL program, this conserved region can form a coiled-coil. Formation of coiled-coil is often responsible for dimerization [Lupas, *Trends Biochem. Sci.*, **21**:375-382 (1996)].

We found that eEF-2 kinases is homologous to the central portion of the recently described MHCKA from *Dictyostelium* [Futey *et al.*, *J. Biol. Chem.* **270**:523-529 (1995) see FIGURE 2]. The kinase was biochemically identified as a 130-kDa protein and has a demonstrated role in myosin assembly, both *in vitro* and *in vivo* [Futey *et al.*, 1995, *supra*]. As with eEF-2 kinase, MHCKA displays no region with detectable similarity to the conserved catalytic domains found in known eukaryotic protein kinases. Primary structure analysis of MHCKA revealed an amino-terminal domain with a probable coiled-coil structure, a central nonrepetitive domain, and a C-terminal domain consisting of seven WD repeats [Futey *et al.*, 1995, *supra*]. A fragment of the central nonrepetitive domain of MHCKA containing amino acids 552-841 was recently shown to represent the catalytic domain [Cote *et al.*, *J. Biol. Chem.* **272**:6846-6849 (1997)].

Because the catalytic domain of MHCKA and eEF-2 kinase have a high degree of similarity, the substrate specificity of these two kinases was assayed. It was demonstrated that MHCKA cannot phosphorylate eEF-2, and likewise, rabbit eEF-2 kinase cannot use myosin heavy chains as a substrate. This demonstrated that each of these kinases is specific for their respective substrates.

EXAMPLE 3eEF-2 KINASE AND MHCKA DEFINE A NEW CLASS OF PROTEIN KINASES

Members of the eukaryotic protein kinase superfamily are characterized by a conserved catalytic domain containing approximately 260 amino acids and is divided into twelve subdomains [Hanks and Hunter, *FASEB J.*, **9**:576-596 (1996); Hardie and Hanks, *The Protein Kinase Facts Book*, Academic, London (1995), Taylor *et al.*, *Annu. Rev. Cell Biol.* **8**:429-462 (1992) Johnson *et al.*, *Cell.* **85**: 149-158 (1996)]. The three-dimensional structure of several protein kinases revealed that the catalytic domain consists of two lobes. The smaller N-terminal lobe, which has a twisted  $\beta$ -sheet structure, represents the ATP-binding domain. The larger C-terminal lobe, which is predominantly  $\alpha$ -helical is involved in substrate binding. At the primary structure level, the only motif similar between eEF-2 kinase, MHCK A, and other protein kinases is the GXGXXG motif which forms the loop interacting directly with the phosphates of ATP [Hanks and Hunter, 1996, *supra*; Hardie and Hanks 1995, *supra*; Taylor *et al.*, *supra*]. In eukaryotic protein kinases, this motif is located at the very N terminus of the ATP-binding lobe of the catalytic domain. In contrast, in a eEF-2 kinase and MHCK A, this motif is close to the C terminus of the catalytic domain (see FIGURE 3). However, the overall topology of the ATP-binding subdomain of eEF-2 kinase and MHCK A can be similar to other protein kinases because the region upstream of the GXGXXG (SEQ ID NO: 21) motif is strongly predicted to contain four or five  $\beta$ -strands and thus can form a twisted  $\beta$ -sheet.

However, the mechanism of ATP-binding to eEF-2 kinase is probably quite different in comparison to other conventional members of the eukaryotic protein kinase superfamily. In protein kinases, there is a conserved lysine residue, corresponding to Lys-72 in cAMP-dependant protein kinases which binds to the  $\beta$ - and  $\gamma$ -phosphates of ATP and is located at about 20 amino acids downstream of the GXGXXG motif. Analysis of eEF-2 kinase and MHCK A sequences revealed that there are no conserved lysine residues in the vicinity of the GXGXXG motif. There is another

atypical protein kinase, BCR-ABLE, which does not contain this conserved lysine and it is proposed that it interacts with ATP *via* two cysteine residues [Maro and Witte, *Cell*, **67**:459-468 (1991)]. Interestingly, eEF-2 kinase and MHCK-A contain two conserved cysteine residues (Cys-313 and Cys-317 in mouse eEF-2 kinase) which are located near the GXGXXG motif and therefore might be involved in ATP binding. Thus the mechanism of ATP-binding of eEF-2 kinase and MHCK A is different from other members of the protein kinase superfamily, but may be similar to that of the BCR-ABLE protein kinase.

The overall catalytic mechanism of eEF-2 kinase and MHCKA is probably also very different from other eukaryotic protein kinases. All members of the eukaryotic protein kinase superfamily contain a DXXXN (SEQ ID NO: 22) motif in the catalytic loop and a DFG motif in the activation segment [Hanks and Hunter, 1996; *supra*, Hardie and Hanks 1995, *supra*; Taylor *et al.*, *supra*; Johnson *et al.*, 1996, *supra*]. These two motifs, which are directly involved in the catalysis of the protein phosphorylation reaction, are absent from the eEF-2 kinase and MHCK A catalytic domain.

We would predict that there are other protein kinases which are structurally similar to eEF-2 kinase and MHCK A. An extensive search of the entire nonrestricted database of the National Center for Biotechnology Information using the BLAST program did not reveal any protein with a significant homology to the catalytic domain of eEF-2 kinase and MHCKA. A search of the Expressed Sequence Tag (EST) database revealed several ESTs from *C. elegans*, mouse and human which are essentially identical to portions of eEF-2 kinase cDNA sequences reported here. Interestingly, a search of the recently completed genome database of *Saccharomyces cerevisiae* did not reveal any protein with homology to eEF-2 kinase despite the fact that eEF-2 phosphorylation was reported in yeast (41).



**Conclusion.** Since the catalytic domains of eEF-2 kinase and MHCK A do not share homology with other known protein kinases, these two protein kinases establish the presence of a novel and widespread superfamily of eukaryotic protein kinases. Although the existence of several unusual protein kinases have been reported, to our knowledge, we demonstrate for the first time the existence of a biochemically well-characterized and ubiquitous protein kinase that is structurally unrelated to other serine/threonine/tyrosine kinases. Contrary to the widely accepted belief that all eukaryotic protein kinases evolved from a single ancestor, our results suggest that eukaryotic protein kinases appeared at least twice during the course of evolution. This also suggests that, in addition to the relatively well-characterized catalytic mechanism employed by members of eukaryotic serine/threonine/tyrosine protein kinase superfamily, there exists another mechanism of protein kinase superfamily, there exists another mechanism of protein phosphorylation. Further studies will reveal the molecular details of this mechanism and whether there are other protein kinases that phosphorylate their substrates using this mechanism.

#### EXAMPLE 4

##### CLONING AND ANALYSIS OF MELANOMA ALPHA KINASE CDNA

Here, we describe cloning and sequencing of a novel protein entitled “melanoma alpha-kinase”. This protein has two domains, one domain is the alpha-kinase catalytic domain and the other is an ion channel. This is the first example of an ion channel being covalently linked to a protein kinase. It is likely that this novel protein kinase can be regulated by ion flow through the membrane. Expression of this kinase was detected in all mouse tissues studied, including heart, skeletal muscle, brain, liver and lung. This kinase is the most abundant in the heart. The ion channel portion is very similar to (70% identical) to a previously identified protein called melastatin that is selectively downregulated in metastatic tumors, and therefore is believed to be a metastasis suppressor gene. Melanoma alpha-kinase, as well as melastatin, belongs to

the TRP family of ion channels. All TRP proteins function as tetramers, and various trp proteins can form tetramers in different combinations that results in ion channel with different properties. Considering the high degree of similarity between melanoma kinase and melastatin, it is likely that melanoma kinase can form tetrameric complexes with melastatin. In humans, melanoma kinase is located on chromosome 15q21.

Human EF-2 kinase amino acid sequence (Acc. No. AAB58270) was used to search for homologous sequences in the expressed sequence tag (EST) division of Genbank using the BLAST server and the tblastn program at the National Center for Biotechnology Information. One human EST (Acc. No. AA332887) that overlapped and displayed significant homology to the catalytic domain of EF-2 kinase was found. We used the nucleotide sequence of this EST to look for overlapping EST sequences. We identified mouse melanoma EST sequence (Acc. No. AA138771) that overlapped by 45 nucleotides with the human EST sequence. We obtained the mouse melanoma EST clone from Research Genetics and sequenced the entire clone. This clone represents the 3' end of melanoma alpha-kinase mRNA and includes the 3' untranslated region plus approximately 350 amino acids of the C-terminus of the protein.

To obtain the full-length cDNA for mouse melanoma alpha-kinase, we used a Marathon-ready mouse heart cDNA library from Clontech. To obtain the remaining sequence of melanoma alpha-kinase, we performed 5' rapid amplification of cDNA ends (RACE) using the following primers: MK1-R1 (5'-TGACCAGGTACACAGCACTTTGACTGCTCT-3' (SEQ ID NO: 23)). PCR was performed under the following conditions: denaturation for 15seconds at 95°C; annealing plus extension for 4 minutes at 68°C, 30 cycles. A single PCR product of approximately 4.0 kb was obtained and gel-purified using a gel extraction kit (Qiagen). The fragment was ligated into vector pCR2.1 using the TA cloning kit (Invitrogen), and then transformed into Escherichia coli TOP10F'. Plasmid DNA was purified, and restriction analysis used to verify the orientation of the coding sequence

with respect to the T7 promoter. Three clones were chosen and sequenced using an ABI 377 sequencer (Applied BioSystems).

To obtain full-length human melanoma alpha-kinase cDNA, new primers were designed for 5' and 3' RACE using sequence information from mouse melanoma alpha kinase cDNA fragments, and full-length cDNA was obtained using a human leukocyte cDNA library (provided by Dr. S. Kotenko).

Mouse melanoma alpha kinase hybridizations were performed using EST 585207 DNA as a probe. The probe was labeled with [ $\alpha$ - $^{32}\text{P}$ ]dCTP using the random-primed DNA labeling method. A multiple tissue northern blot (CLONTECH) was prehybridized at 42°C for 16 hours in a 50% formamide solution containing 10 $\times$  Denhardt's solution, 5 $\times$  SSPE, 2% SDS, and 100  $\mu\text{g/ml}$  salmon sperm DNA. Hybridizations were completed in the same solution containing the  $^{32}\text{P}$ -labeled probe (1  $\times$  10<sup>6</sup> cpm/ml; specific activity, 1  $\times$  10<sup>8</sup> dpm/ $\mu\text{g}$  DNA) and 10% dextran sulfate at 42°C for 16 hours. Blots were washed twice at room temperature (15 minutes) in 2 $\times$  SSPE, 0.05% SDS, and once at 50°C (15 minutes) in 0.5 $\times$  SSPE, 0.5% SDS. RNA/cDNA hybrids were visualized by autoradiography.

### EXAMPLE 5

#### A NOVEL TYPE OF SIGNALING MOLECULE-PROTEIN KINASES COVALENTLY LINKED TO ION CHANNELS

##### ABSTRACT

Recently we identified a new class of protein kinases with a novel type of catalytic domain structurally and evolutionarily unrelated to the conventional eukaryotic protein kinases. This new class, which we named alpha-kinases, is represented by eukaryotic elongation factor-2 kinase and the Dictyostelium myosin heavy chain kinases. Here we cloned and sequenced five other mammalian alpha-kinases. One of these proteins, which was initially identified as an EST from a mouse melanoma

cDNA library, was named melanoma alpha-kinase (MK), and according to northern analysis, has a ubiquitous tissue distribution, being present in all mouse and human tissues studied. Four other alpha kinases have a more restricted tissue distribution and were named after the tissue in which they are predominantly expressed: kidney alpha-kinase (KK), heart alpha-kinase (HK), skeletal muscle alpha-kinase (SK), and lymphocyte alpha-kinase (LK). All these protein kinases are large proteins of more than 1000 amino acids with a typical alpha-kinase catalytic domain located at the very carboxyl-terminus. We expressed the catalytic domain of human MK in *Escherichia coli*, and found that it autophosphorylates on threonine residues, demonstrating that it is a genuine protein kinase.

Unexpectedly, we found that the long amino-terminal portions of melanoma and kidney  $\alpha$ -kinases represent new members of the transient receptor potential (TRP) ion channel family, which are implicated in the mediation of capacitative  $\text{Ca}^{2+}$  entry in non-excitabile mammalian cells. This suggests that melanoma and kidney  $\alpha$ -kinases, which represent a novel type of signaling molecule, are involved in the regulation of  $\text{Ca}^{2+}$  influx into mammalian cells. It has also been implied that TRP channels may mediate the  $\text{Ca}^{2+}$ -release-activated  $\text{Ca}^{2+}$  current (CRAC). The channel portions of KK and MK were highly similar to each other and highly similar to melastatin.

Melastatin is a putative  $\text{Ca}^{2+}$  channel that was identified as a gene product specifically downregulated in metastatic melanoma. Phylogenetic analysis revealed that both KK and MK belong to the long TRP (LTRP) channel subfamily, which also includes melastatin, and several uncharacterized channel proteins from mammals, *Caenorhabditis elegans* and *Drosophila*. Among LTRP channels, only MK and KK possess an  $\alpha$ -kinase domain.

## INTRODUCTION

The vast majority of eukaryotic protein kinases have a typical catalytic domain structure consisting of twelve conserved subdomains (1). The existence of other protein kinases with a different structure was reported in eukaryotes (2-4). Recently

we identified a new class of protein kinases with a novel type of catalytic domain, structurally and evolutionarily unrelated to the conventional eukaryotic protein kinases (5). This class, which we named alpha-kinases, is represented by eukaryotic elongation factor-2 (eEF-2) kinase and the Dictyostelium myosin heavy chain kinases A and B (MHCKA and B) (2,3,29). The catalytic domain of the alpha-kinases can be subdivided into eight domains (6). There is no significant homology between those eight domain and any of the twelve subdomains of the conventional protein kinases. We named this new class of protein kinases the alpha-kinases because the existing evidence suggests that they can phosphorylate amino acids located within  $\alpha$ -helices (6).

In order to study how widespread the alpha-kinases are and to identify new members of the alpha-kinase family in mammals, we used a functional genomic approach. We performed an extensive expressed sequence tag (EST) database search for sequences homologous to the catalytic domain of human eEF-2 kinase. As a result of this screen, we obtained several partial sequences for putative alpha-kinases, which were subsequently used to clone the full-length cDNAs.

We cloned and sequenced and analyzed the tissue distribution of five new members of the alpha-kinase family. All these proteins contain the typical alpha-kinase catalytic domain. The expressed catalytic domain of MK was able to autophosphorylate, therefore demonstrating that it is a genuine protein kinase.

Unexpectedly, the amino-terminal portions of MK and KK appear to have a long amino-terminal portions highly homologous to a number of ion channel proteins that belong to transient receptor potential (TRP) family of  $\text{Ca}^{2+}$  channels. The ion channel portions of MK and KK are remarkably homologous to melastatin. Melastatin is a protein downregulated in metastatic melanoma, and is a newly discovered member of the TRP  $\text{Ca}^{2+}$  channel family (7, 8). TRP channels derive their name from a mutation in a *Drosophila* calcium channel that is involved in

photoreception (30,31). This mutation caused an inability to maintain a sustained receptor potential, and was therefore named transient receptor potential (*trp*), and the channel was named the TRP channel (19,30,31). Several homologues of TRP channels in mammals have been identified (27,32,33). The recent interest in the TRP channel family is related to the fact that they may represent channels responsible for store-operated calcium influx – one of the major pathways of calcium entry into non-excitable mammalian cells (9,10,12,18,24,34).

TRP channels are  $\text{Ca}^{2+}$ -permeable channels believed to be responsible for  $\text{Ca}^{2+}$  influx in response to depletion of internal  $\text{Ca}^{2+}$  stores (9-11). The ion channel portion of MK and KK, being highly similar to melastatin, makes MK and KK new member of the TRP family, in particular the LTRP family to which melastatin belongs.

Thus, in our work, we demonstrated a novel type of signaling molecule - an ion channel covalently linked to a protein kinase. We discuss the possibility that this hybrid ion channel/protein kinase is involved in the regulation of store-operated  $\text{Ca}^{2+}$  entry in non-excitable tissues.

## MATERIALS AND METHODS

### Cloning of Melanoma Kinase

We searched the EST database, and identified several mouse and human ESTs homologous to the catalytic domain of human eEF-2 kinase. One of these EST clones was derived from a mouse melanoma cDNA library (IMAGE clone 585207; GenBank accession #AA138771). We sequenced this clone and found it encodes the C-terminus (approximately 300 amino acids) of a novel protein. We used 5' rapid amplification of cDNA ends (RACE) and a mouse heart Marathon-ready cDNA library (Clontech) to determine the full-length sequence. We used this sequence information to design primers and clone human melanoma kinase from a HeLa cell cDNA library.

### Cloning of Kidney Kinase

A further search in the EST database revealed an EST derived from a mouse kidney cDNA library encoding another protein homologous to the catalytic domain of eEF-2 kinase (IMAGE clone 656119; GenBank accession # AI390333). We used the sequence information to perform 5'RACE and 3'RACE using a mouse heart Marathon-ready cDNA library (Clontech). After partial sequencing of this clone, we used the new sequence information to design primers and clone human kidney kinase from a human kidney Marathon-ready cDNA library (Clontech).

### Cloning of Heart Kinase

Database searches revealed another homologous EST clone approximately 2 kb in length (IMAGE clone #585879; GenBank accession #AA140393). To obtain the full length cDNA, we screened a mouse heart 5'-STRETCH PLUS cDNA lambda library (Clontech). A 32P-labeled 2kb EST fragment was used as a probe for clone identification. Several clones gave positive signals, and were further analyzed by PCR. The largest of the clones (~5 kb) was sequenced. Subsequently we found a human EST in the EST database homologous to mouse heart kinase (IMAGE clone #843057; GenBank accession #AA485987). We sequenced this clone, and found it encodes a protein corresponding to the C-terminus of heart kinase. After partial sequencing of this clone, we used the new sequence information to design primers and clone human heart kinase from a human heart Marathon-ready cDNA library (Clontech).

### Cloning of Skeletal Muscle Kinase

We searched the HTGS database for sequences homologous to mouse heart kinase, and found a clone containing the gene encoding a protein similar to heart kinase. We designed primers using the database sequence and performed PCR using a human placenta cDNA library to clone the catalytic domain of muscle kinase.

### Cloning of Lymphocyte Kinase

By searching the non-restricted database, we found a genomic DNA clone derived from chromosome IV that encodes a protein containing the  $\alpha$ -kinase catalytic domain. We used this sequence information to search for overlapping ESTs in order to reconstruct the full-length protein, and then to design primers for PCR to clone the full-length protein from a lymphocyte cDNA library.

#### Cloning of the melanoma kinase catalytic domain

The catalytic domain of melanoma kinase was cloned from a HeLa cell cDNA library. Primers were designed based upon the sequence of melanoma kinase. The sequences of the primers are:

Forward: 5'- GTTAGTACACCATCTCAGCCAAGTTGCAAA-3', (SEQ ID NO: 24)

Reverse: 5'-TTATAACATCAGACGAACAGAATTAGTTGATTCTGATTCT-3'  
(SEQ ID NO: 25).

PCR conditions were as follows: 30 sec. at 94°C, 30 sec. at 58°C, 3 min. at 72°C for 30 cycles, followed by a 10 min. final extension at 72°C. The PCR product was cloned into PCRII-TOPO vector (Invitrogen) as per manufacturer's instructions. The insert was then subcloned into the EcoRI site in pMAL-p2x (NEB) to tag the protein with maltose binding protein (MBP).

#### Expression and purification of MBP-MK

E. coli strain DH5 $\alpha$  carrying the pMALp2x-MK plasmid were grown to an OD<sub>600</sub>=0.5, then IPTG was added to a final concentration 0.3mM. Cells were grown for additional 6 hours at 37°C. All following procedures were carried out at 4°C. Cells were resuspended in 20mM Tris-HCl (pH 7.4), 200mM NaCl, 1mM EDTA, 10mM  $\beta$ -mercaptoethanol and sonicated. Inclusion bodies were pelleted by centrifugation at 30,000xg for 30 min., dissolved in 6M urea, 20mM Tris-HCl (pH 7.4), 200mM NaCl, 1mM EDTA, 10mM  $\beta$ -mercaptoethanol, 20% (w/v) glycerol and centrifuged again at 30,000xg for 30 min. The supernatant was dialyzed overnight against the same buffer but without urea. After dialysis, the sample was centrifuged once again at 30,000xg for 30 min., and the supernatant was loaded onto an amylose column equilibrated with



20mM Tris-HCl (pH 7.4), 200mM NaCl, 1mM EDTA, 10mM  $\beta$ -mercaptoethanol, 20% (w/v) glycerol. Elution was performed by a step gradient of the same buffer plus 10mM maltose.

#### Phosphorylation of MBP-MK

Assays for MBP-MK activity were performed at 30°C in an assay buffer containing 50mM Hepes-KOH (pH 6.6), 10mM MgCl<sub>2</sub>, 1mM DTT, 50mM ATP, 20mCi [g-<sup>32</sup>P]-ATP and 3mg MBP-MK. After incubation, Laemmli sample buffer was added, samples were boiled and 25ml of each sample was loaded onto a 10% SDS-PAGE gel. The gel was stained with Coomassie Blue, dried and exposed to film for 16 hours.

#### Phosphoamino acid Analysis

Phosphorylation of MBP-MK was done as described above, but the reaction volume was increased 5-fold. The incubation time was 2 hours. Samples were separated by 10% SDS-PAGE and transferred to an Immobilon-P membrane (Millipore). The portion of membrane with phospho-MBP-MK was excised and incubated in 6M HCl at 110°C for 1 hour. After incubation, the mixture was dried, and the dried pellet was dissolved in 9 $\mu$ l of water, with the addition of non-radioactive phosphoserine, phosphothreonine, and phosphotyrosine. Phosphoamino acids were separated by thin-layer chromatography on cellulose (cellulose on polyester; Aldrich) using a buffer consisting of isobutyric acid and 0.5M NH<sub>4</sub>OH in a 5:3 ratio. The TLC plate was stained with 0.2% ninhydrin and exposed to film.

#### Northern Blot Analysis

Standard Multiple Tissue Northern (MTN) Blots (Clontech) were stained with DNA probes according to manufacturer's instructions. The probes were labeled with [ $\alpha$ -<sup>32</sup>P]-dCTP using the Megaprime DNA labeling system (Amersham) using specific DNA fragments as templates. The DNA fragments were obtained as follows.

A 430 bp DNA fragment of human melanoma kinase (corresponding to nucleotides 4331-4761) was obtained by PCR from a Hela cDNA library (Clontech). The following primers were used for PCR:

LMK2: 5'CTGCGACAGAGACTACATGGGGTAGAACTC 3'(SEQ ID NO: 42)

LMK4: 5'TGAGTGTCTTCGGTAGATGGCCTTCTACTG 3'(SEQ ID NO: 43)

A 741 bp DNA fragment of human kidney kinase was produced by PCR from a plasmid containing kidney kinase cDNA. The region corresponding to nucleotides 3721-4462 was amplified using the following primers:

KK-F1: 5'ATGGAGATTGCTGGAGAGAAG 3' (SEQ ID NO: 44) and

KK-R3: 5'ATCACTACTCTGGGCCGATC 3'(SEQ ID NO:45)

A 1.2 kb DNA fragment of human muscle kinase was obtained by *Eco*RI digestion of the human muscle kinase cDNA insert cloned in pCRII-TOPO (Invitrogen). The mouse melanoma kinase 2.2 kb DNA fragment was obtained from EST clone 585207. The fragment was cut out with *Bam*HI and *Xho*I from pBluescript SK- vector (Stratagene). The mouse heart kinase 2 kb DNA fragment was produced by restriction with *Sma*I and *Kpn*I from EST clone 585879.

## RESULTS

In our work we cloned and sequenced five new members of the alpha-kinase family. They are named according to their tissue distribution: heart alpha-kinase (HK), melanoma alpha-kinase (MK), kidney alpha-kinase (KK), skeletal muscle alpha-kinase (SK), lymphocyte alpha-kinase (LK). The nucleic acid sequence and predicted amino acid sequence of the human heart alpha-kinase (HK) are depicted in FIGURE 8 A and B (SEQ ID NO: 34 and 35, respectively). The nucleic acid sequence and predicted amino acid sequence of the mouse heart alpha-kinase (HK) are provided in SEQ ID NO: 36 and 37, respectively. The nucleic acid sequence (SEQ ID

NO: 26) and predicted amino acid sequence (SEQ ID NO: 27) of the human melanoma alpha-kinase (MK) are depicted in FIGURE 7 A and B. The nucleic acid sequence (SEQ ID NO: 28) of mouse melanoma  $\alpha$ -kinase is shown in FIGURE 5. The predicted amino acid sequence (SEQ ID NO: 29) of mouse melanoma  $\alpha$ -kinase is shown in FIGURE 6. The nucleic acid and predicted amino and sequence of the human kidney alpha-kinase (KK) are depicted in FIGURE 9 A and B (SEQ ID NO: 30 and 31, respectively). The nucleic acid and predicted amino and sequence of mouse kidney alpha-kinase (KK) are provided in SEQ ID NO: 32 and 33, respectively. FIGURE 10 A and B depicts the nucleic acid sequence (SEQ ID NO: 38) and predicted amino acid sequence (SEQ ID NO: 39) of human skeletal muscle alpha-kinase (SK). The nucleic acid sequence (SEQ ID NO: 40) and predicted amino acid sequence (SEQ ID NO: 41) of the lymphocyte alpha-kinase (LK) are shown in FIGURE 11 A and B. All of these protein kinases are large proteins of more than 1000 amino acids with a typical alpha-kinase catalytic domain located at the very C-terminus. FIGURE 12 shows the alignment of the catalytic domains of the cloned alpha-kinases. The catalytic domain sequence reveals 30-80% similarity between these alpha-kinases. It can be divided into several subdomains with no homology between these subdomains and any of the twelve subdomains of the conventional protein kinases. Altogether, there are sixteen positions in the alignment of the cloned proteins that are invariant among all the known alpha-kinases.

All five new proteins are homologous to each other, as well as homologous to the eEF-2 kinase and MHCK B catalytic domains. A comparison of the five new  $\alpha$ -kinases, eEF-2 kinase and MHCK B reveals sixteen invariant amino acids. We reported previously (6) that the  $\alpha$ -kinase catalytic domain can be divided into eight subdomains, each having a characteristic sequence motif. Identification of new  $\alpha$ -kinases allowed us to characterize these subdomains more precisely. In addition, we used the ALB secondary structure prediction service (<http://indy.ipr.serpukhov.su/~rykunov/alb/>) (35) to predict a consensus secondary structure of the  $\alpha$ -kinase catalytic domain. Subdomain I begins with a conserved Trp residue present in all  $\alpha$ -kinases with the exception of HK, which has a Phe in this

position. Subdomain I is also characterized by an invariant Gly and an invariant Arg that are part of the conserved Arg-Lys-Ala motif. Subdomain II is characterized by an invariant Lys, that is part of an Hyd-Hyd-X-Lys motif (Hyd = hydrophobic). Subdomain III contains an invariant Gln, and is predicted to form an  $\alpha$ -helix. Subdomain IV contains a stretch of hydrophobic amino acids and is predicted to form a  $\beta$ -sheet. Subdomain V is predicted to form an  $\alpha$ -helix containing an invariant Glu, and is followed by a conserved Asn (an Arg in HK and SK). Subdomain VI is predicted to form a  $\beta$ -turn- $\beta$ -turn structure with an invariant His in the first  $\beta$ -strand, and the conserved sequence, Leu-Leu-Val-Val-Asp-Leu-Gln-Gly, that forms the end of the second  $\beta$ -strand and second turn and also contains an invariant Asp and Gly. Subdomain VII is characterized by the conserved sequence, Leu-Thr-Asp-Pro-Gln-Ile, which contains an invariant Thr-Asp. Subdomain VIII begins with a Gly-rich region that is not predicted to have any regular secondary structure, followed by a sequence containing invariant Phe and His residues, an invariant Cys-Asn-X-X-Cys motif and an invariant Leu. The region containing the Cys-Asn-X-X-Cys motif is predicted to form a short  $\alpha$ -helix.

Phylogenetic analysis (FIGURE 13) of the cloned alpha-kinases suggests that all five alpha-kinases are more closely related to each other than to eEF-2 kinase or the MHCKs, and form a separate subfamily. MK is closely related to KK (78% identity). HK is similar to SK (47% identity). LK has less similarity to the others, but they all form a distinctive separate subfamily of alpha-kinases, displaying various degrees of similarity to eEF-2 kinase.

In order to analyze kinase activity, we expressed the catalytic domain of MK as maltose-binding protein (MBP) fusion protein. Affinity-purified MBP-MK was able to autophosphorylate. FIGURE 14 shows the time course of  $^{32}\text{P}$  incorporation into MBP-MK. This phosphorylation can be reversed by incubation with lambda phosphatase. Phosphoamino acid analysis revealed that MK is phosphorylated on a threonine residue.

Using northern blot analysis, we analyzed the tissue distribution of the a-kinases in human and mouse tissues (FIGURE 15). MK, KK, HK and SK are large proteins (corresponding cDNAs are 7.5kb). LK mRNA is represented by two bands, 5.5 and 7.5 kb. An additional minor band at 9.5 kb can be seen for SK. MK is ubiquitously expressed, being detected in every tissue tested. In human tissues, it is most abundant in the liver, kidney and heart, and in mouse tissues - heart, lung, liver and kidney. There were noticeable amounts in human lymphoid, bone marrow and thymus tissues. The least amount of MK mRNA, among human tissues, was observed in brain. LK mRNA can be detected by northern analysis in various human tissues. Its tissue distribution was virtually identical to MK, although it is much less abundant than MK since three weeks of exposure were required to visualize the bands. LK can be detected by reverse transcriptase-PCR (RT-PCR) in human fetal liver and placenta tissues, and lymphocyte libraries. A full-length clone was obtained from a lymphocyte cDNA library. KK is present almost exclusively in kidney tissue, with trace amounts in human lymphocyte, brain and bone marrow tissues. HK is found almost exclusively in mouse heart tissue, with a smaller amount in skeletal muscle tissue. It was not seen in any other tissues tested. SK is very abundant in human muscle tissues, with a considerable amount in human heart tissue. Trace amounts of SK can be seen in human lung, placenta and kidney tissues.

We cloned full-length cDNA for MK and KK. The long N-terminal portion of MK and KK display high similarity to ion channels homologous to TRP channel family. The MK sequence contained 1864 amino acids and the KK sequence contained 2011 amino acids. Unexpectedly, we found that the long amino terminal portions of MK and KK are homologous to ion channels. The approximately 1200 amino acid long N-terminal portions of MK and KK were similar to each other (59% identical) and homologous to melastatin (48% and 51% identical respectively; see FIGURE 17). Melastatin is a putative  $\text{Ca}^{2+}$  channel that belongs to the transient receptor potential (TRP) family of ion channels (8). Like melastatin, MK and KK contain all the sequence elements characteristic of the TRP channel family. These elements include

six predicted transmembrane segments, a highly conserved sequence in the putative pore region between transmembrane segments 5 and 6, a highly conserved sequence at the end of transmembrane segment 6, and a Pro-Pro-Pro motif-containing sequence that immediately follows transmembrane segment 6 (FIGURE 17). We found several human, mouse, *Caenorhabditis elegans* and *Drosophila* proteins in GenBank that are highly similar to the melastatin-like portions of MK and KK (FIGURE 17). All these proteins belong to a subfamily of the TRP channels which were named long TRP channels (LTRPC), and are characterized by a long conserved N-terminal sequence that precedes the transmembrane segments (14). These proteins include a protein that was initially called TRPC7 (36), and was later renamed LTRPC2 (14), a protein named MTR1 or LTRPC5 (14,37), an unnamed human putative protein we named LTRPC6 (GenBank accession #AK000048), three *C. elegans* proteins (F54D1.5, T01H8.5, and C05C12.3) named respectively CeLTRPC1, CeLTRPC2 and CeLTRPC3 (14), and a *Drosophila* putative protein that we named DmLTRPC1 (GenBank accession #AE008311) (FIGURE 17). Thus, MK and KK can be classified as members of the LTRPC subfamily, and we suggest they be designated LTRPC3 and LTRPC4, respectively. As can be seen in FIGURE 17, MK and KK, like other LTRPC proteins, contain a long N-terminal sequence (approximately 600-800 amino acids) that has several conserved and unique motifs. The ALB program predicted several long  $\alpha$ -helices in this region in all LTRPC proteins.

Phylogenetic analysis revealed that MK, KK as well as other LTRPC proteins are related to the prototypic *Drosophila* TRP protein, but are more similar to each other than to the prototypic TRP (FIGURE 19).

We also determined the full-length sequence of LK cDNA that encodes a protein containing 1242 amino acids. The TMPred program predicts four transmembrane segments located close to the N-terminus.

HK and SK cDNAs encode proteins of 1531 and 1215 amino acids, respectively. Comparison of the predicted amino acid sequences of HK and SK with other proteins in GenBank using the BLAST program revealed that there is an approximately 100 amino acid motif located just N-terminal to the catalytic domain that displays sequence similarity to immunoglobulin-like domains of titin, myosin light chain kinase, and several other proteins. The same region in both kinases is identified as an immunoglobulin-like domain using the CD-Search program. The remainder of the HK amino acid sequence did not display any strong similarity to any known proteins. The N-terminal portion of SK displayed a weak similarity to collagen, which may be attributed to the high glycine and proline content.

## DISCUSSION

In our previous work, we discussed the unique structure of eEF-2 kinase and the *Dictyostelium* MHCKs, and suggested that they represent a new class of protein kinases, the  $\alpha$ -kinases (5,6). In this work, we cloned and sequenced five new members of the  $\alpha$ -kinase class. Thus, together with eEF-2 kinase, there are at least six distinct  $\alpha$ -kinases in mammals. These six  $\alpha$ -kinases encompass all sequences from vertebrate sources deposited in GenBank thus far with homology to the  $\alpha$ -kinases. With approximately 90% of the human genome represented in GenBank to date, it is likely we cloned most, if not all, of the mammalian  $\alpha$ -kinases. Interestingly, in the *C. elegans* genome there is only one  $\alpha$ -kinase (eEF-2 kinase) while none have been identified in the *Drosophila*, yeast and plant genomes. However, the  $\alpha$ -kinases appear to be widespread among the protozoa: sequences encoding proteins with a high similarity to the  $\alpha$ -kinases are present in the genomes of *Trypanosoma*, *Leishmania*, and *Amoeba*.

We cloned and sequenced five new members of the alpha-kinase family. All of these kinases have a typical alpha-kinase catalytic domain located the very carboxyl-terminus. The alignment of the cloned alpha-kinase catalytic domains

reveals eight subdomains characteristic of the  $\alpha$ -kinases which have no significant homology to the conventional eukaryotic protein kinase catalytic domain. An alignment of the  $\alpha$ -kinase catalytic domain revealed several characteristic motifs. These motifs are different from those that characterize the eukaryotic Ser/Thr/Tyr protein kinase superfamily, suggesting that the  $\alpha$ -kinases and conventional eukaryotic protein kinases are structurally and evolutionarily unrelated. The expressed catalytic domain of MK is able to efficiently autophosphorylate. Tissue distribution of the new cloned proteins reveals that among the cloned  $\alpha$ -kinases, MK has a wide tissue distribution, while the others (KK, HK, LK and SK) are specific for particular tissues. SK, which is specific to human skeletal muscle, is remarkably abundant. Phylogenetic analysis suggests that our cloned  $\alpha$ -kinases are closely related to each other, and are distantly related to eEF-2 kinase and the MHCKs, forming a distinctive subfamily of  $\alpha$ -kinases. All of the five new  $\alpha$ -kinases probably evolved from a common ancestor during the evolutionary process. Therefore, the  $\alpha$ -kinase family has been enlarged and now includes five new members.

Recently the structure of a novel type of protein kinase catalytic domain has been determined represented by the bacterial histidine kinases, EnvZ and CheA (38,39). The structure of the catalytic domain of the histidine kinases appears to be completely different from that of the Ser/Thr/Tyr protein kinase superfamily but utilizes a fold similar to Hsp90, DNA gyrase B, and MutL (Bergerat fold; 40). There are protein kinases that are highly similar to the bacterial histidine kinases, but phosphorylate their substrates on serine residues [for example, plant phytochromes (41) and animal pyruvate dehydrogenase kinase (42)] or on tyrosine residues [DivL (43)], suggesting that protein kinases with the Bergerat fold can phosphorylate amino acids other than histidine. Is it possible that the  $\alpha$ -kinases also use a similar fold? We noticed that the distribution of consensus secondary structure elements predicted for the  $\alpha$ -kinases using the ALB program is similar to the distribution of secondary structure elements in EnvZ as determined by NMR. Moreover, the conserved asparagine and invariant aspartic acid residues located in subdomains V and VI, respectively, may correspond



to the invariant asparagine and aspartic acid residues located in the N and G1 boxes, respectively, of the histidine kinases. These residues play a crucial role in ATP binding (38,39,40). The glycine-rich region in subdomain VIII of the  $\alpha$ -kinases may correspond to the G2 box of histidine kinases, which is a highly mobile region that forms part of the "lid" of the ATP binding site (38,39,40). In addition, the histidine residues phosphorylated by histidine kinases are located within  $\alpha$ -helices (39,44,45), suggesting that the catalytic domain of these protein kinases is adapted to recognize  $\alpha$ -helices. Finally, the overall topology of some  $\alpha$ -kinases (MK, KK and LK) with several transmembrane segments in the N-terminal region of the molecule and catalytic domain located at the C-terminal region resemble the topology of many histidine kinases (46). All these facts raise the possibility that the  $\alpha$ -kinases and the histidine kinases may be evolutionarily related.

The expressed catalytic domain of MK is able to autophosphorylate, demonstrating that it is a genuine protein kinase. Phosphoamino acid analysis revealed that MK autophosphorylates exclusively on a threonine residue. Interestingly, the only two other  $\alpha$ -kinases for which the substrates have been identified, eEF-2 kinase and MHCK A, both phosphorylate their substrates on threonine residues. Therefore, it is possible that the  $\alpha$ -kinases in general are specific for phosphothreonine.

Northern analysis reveals that MK and LK have a wide tissue distribution suggesting a general function for these kinases, while KK, HK and SK are expressed primarily within specific tissues, suggesting they have tissue-specific functions. Interestingly, the tissue distribution patterns of MK and LK were virtually identical, suggesting that these two proteins are similarly regulated and may have similar functions.

Remarkably, two of the new members, MK and KK, appeared to have ion channels at the very amino-terminus that are highly homologous to the TRP channel family. TRP channels are  $\text{Ca}^{2+}$ -permeable channels that are believed to be responsible for  $\text{Ca}^{2+}$  influx in response to depletion of internal  $\text{Ca}^{2+}$  stores (9-11). Such a depletion of

intracellular  $\text{Ca}^{2+}$  stores followed by activation of the  $\text{Ca}^{2+}$  entry mechanism at the plasma membrane is called capacitative  $\text{Ca}^{2+}$  entry (CCE).

CCE is loosely defined as an influx of  $\text{Ca}^{2+}$  from the extracellular space following inositol 1,4,5-triphosphate (IP3) or other  $\text{Ca}^{2+}$ -mobilizing agent-induced depletion of internal  $\text{Ca}^{2+}$  from the ER and SR. CCE plays a central role in many aspects of cell signaling, and is present in many types of cells. It is an essential component of the cellular response to many hormones and growth factors. (12, 13). The TRP gene of *Drosophila* (19, 20) and its homologue, TRP-like (TRPL; 21) together with recently-discovered mammalian homologues (22-25) were suggested to encode the CCE channels (26, 27). The family of known TRPs and their homologues are conserved from worms to humans. All show the same basic channel subunit structure with six putative transmembrane helices, and a range of motifs in the amino- and carboxyl-terminal regions (14).

It was recently suggested that by their functional properties and structure, the TRP channel family can be subdivided into three subfamilies: short TRP (STRP), osmoTRP (OTRP) and long TRP (LTRP) (14). MK and KK display similarity to melastatin, which, by its structure and function, can be classified as an LTRP (14). Melastatin is a putative  $\text{Ca}^{2+}$  channel identified recently as a gene specifically downregulated in metastatic melanoma (8). Sequence analysis of the ion channel region of MK and KK reveals a typical structure of LTRPs. The function of STRP including *Drosophila* TRP and many mammalian homologs is to mediate  $\text{Ca}^{2+}$  influx subsequent to activation of phospholipase C. The OTRP subfamily is  $\text{Ca}^{2+}$ -permeable channels involved in pain transduction, chemo-, mechano- and osmoregulation. The function of the LTRP subfamily channels is not yet well characterized.

LTRP channels have longer coding sequences than STRP and OTRP, particularly at their amino-termini. All TRP channels share similar structure: they have six

transmembrane segments and a pore-forming loop between the fifth and sixth segments (reviewed in 24,9,14,47). The same conserved sequences are present in MK and KK (fig. 4). The Pro-Pro-Pro motif that follows the sixth transmembrane segment in MK and KK is characteristic for STRP and LTRP channels. LTRPs do not have the ankyrin repeats characteristic of the STRPs and OTRPs (14). Instead, they have a long N-terminus with a unique and highly-conserved sequence. As can be seen in FIGURE 17, the long N-terminal portion of MK and KK also has this highly conserved sequence. This region in MK and KK is predicted to form several long  $\alpha$ -helices. In addition to MK and KK, we identified eight other LTRP channels among the sequences deposited in GenBank: four in mammals, three in *C. elegans*, and one in *Drosophila*. The first LTRP to be identified was melastatin, a putative  $\text{Ca}^{2+}$  channel whose mRNA is specifically downregulated in metastatic melanoma (7,8). MK and KK are particularly similar to melastatin (more than 48% and 51% identity) suggesting that MK and KK may be a product of a recent evolutionary event – a fusion between the  $\alpha$ -kinase catalytic domain and a melastatin-like ion channel. Thus, considering the striking similarity of MK and KK to LTRP channels, we suggest that MK and KK are ion channels with a unique molecular structure – ion channels covalently linked to a protein kinase. FIGURE 18 and FIGURE 19 show a schematic representation of the major domains of MK, KK as well as other  $\alpha$ -kinases, a phylogenetic tree of LTRP channels, and a proposed structural model of MK and KK. Thus, MK and KK, having ion channel part with the typical structure of LTRPs, can be considered a new member of the LTRP family. To date, MK and KK are the only known channels covalently linked to a protein kinase catalytic domain.

It is possible that the other three  $\alpha$ -kinases may also have regions homologous to ion channels. LK has four predicted transmembrane segments near its N-terminus, although this region is not similar to the TRP channels. We did not find sequences homologous to ion channels at the N-termini of HK and SK, however, it is likely that we did not obtain the very N-terminal regions of these proteins.

TRP channels are  $\text{Ca}^{2+}$ -permeable channels believed to be responsible for  $\text{Ca}^{2+}$  influx in response to depletion of internal  $\text{Ca}^{2+}$  stores (9-12,18,24,27,32,34). The TRP gene of *Drosophila* (19,31) together with recently-discovered mammalian homologues (27,32,33) were suggested to encode store-operated  $\text{Ca}^{2+}$  channels, also known as capacitative  $\text{Ca}^{2+}$  entry channels (9,10,12,18,24,27,31,32,34).

The first of the  $\text{Ca}^{2+}$ -permeable store-operated channels to be characterized in detail were those mediating  $\text{Ca}^{2+}$ -release-activated current ( $I_{\text{CRAC}}$ ) (16,48,51). CRAC channels are highly  $\text{Ca}^{2+}$  selective, low conductance channels that mediate  $\text{Ca}^{2+}$  entry in response to depletion of  $\text{Ca}^{2+}$  from intracellular stores in various non-excitable cells, and play a central role in activation of lymphocytes, degranulation of mast cells, and possibly mitogenic stimulation of various cells (16,48,51,54,55). The molecular identity of CRAC channels has not yet been determined. It has been suggested that members of the TRP channel family may underlie  $I_{\text{CRAC}}$  (9,10,12,18,24,27,32,34,55). However, none of the TRP channels studied to date have all the properties of CRAC channels (14). Nevertheless, TRP proteins are currently the most likely candidates for CRAC channels, and it has been suggested that the CRAC channels may be hidden in the LTRP family whose function is largely unknown (14).

It is possible that MK and KK are indeed the "hidden" members of the LTRP channel family mediating  $I_{\text{CRAC}}$ . The tissue distribution of MK (which is ubiquitously expressed in all tissues tested, and predominant in non-excitable tissues such as liver and lymphocytes) is consistent with this idea. Moreover, the protein kinase domain can be part of the signaling mechanism that modulates channel function. There is evidence that protein phosphorylation is involved in both the activation of the store-operated  $\text{Ca}^{2+}$  channels as well as in the regulation of channel closure (54,56-59).

The conserved location of the transmembrane domain and catalytic domains linked together reveals a new structure for a novel type of protein. What is the role of such an unusual protein structure? There are a number of reports indicating a role for

protein phosphorylation in CCE. There are indications that protein phosphatases may regulate the responsiveness of the entry channel to hypothetical diffusible component of the entry, implying that the latter may act by promoting channel phosphorylation (34). It has been proposed that the endoplasmic reticulum might possess protein kinases or phosphatases capable of altering the phosphorylation state of the entry channel (12). Phosphatase inhibitors will enhance  $\text{Ca}^{2+}$  entry by serine/threonine phosphorylation (52). Tyrosine phosphorylation has been implicated in coupling store depletion of  $\text{Ca}^{2+}$  entry, but there are inconsistencies in overall information and a possible explanation is that separate kinases phosphorylate different components of the entry mechanism. Different kinases may be involved in the process of CCE, one of the consistent implications is that protein kinases possibly phosphorylate the IP3 receptor, but the CRAC channel is the likely target for protein phosphorylation (12). It was also shown that phosphatase inhibitors can inhibit ICRAC. The tissue distribution of MK is consistent with its being a CRAC since it is present in all tissues and is most prominent in non-excitables, while barely detectable in the brain. We suggest that we discovered a new type of molecule - a protein kinase covalently linked to an ion channel - represents a new signaling molecule which underlies CRAC channels. The placement of a kinase and channel on a single molecule is particularly interesting and suggests a self-regulated molecule, whereby the phosphorylation/autophosphorylation of these unique alpha kinases controls or contributes to the open or closed state of the channel. In addition, such an unusual molecular structure may be a part of a signal transduction mechanism that links depletion of internal  $\text{Ca}^{2+}$  stores to channel opening.

In summary, our discovery of five new members has broadened the class of  $\alpha$ -kinases. Two of the new  $\alpha$ -kinases represent a novel type of signaling molecule - a TRP-like ion channel covalently linked to a protein kinase suggesting that one of the functions of the  $\alpha$ -kinases is to regulate  $\text{Ca}^{2+}$  influx in mammalian cells. It is also possible that MK and KK are CRAC channels and play a central role in the immune response.

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This invention may be embodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure is therefore to be considered as in all aspects illustrate and not restrictive, the scope of the invention being indicated by the appended Claims, and all changes which come within the meaning and range of equivalency are intended to be embraced therein.

Various references are cited throughout this Specification, each of which is incorporated herein by reference in its entirety.